Spécificité de la coopération phytostimulatrice Azospirillum-céréales

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Spécificité de la coopération phytostimulatrice *Azospirillum*-céréales

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Résumé

La spécificité d'hôte est un concept fondamental pour comprendre les mécanismes évolutifs ayant abouti aux interactions étroites entre les bactéries et les plantes. Les études réalisées sur la coopération entre les bactéries rhizosphériques phytostimulatrices (PGPR) et les plantes suggèrent que la spécificité serait régie soit par une adaptation souche-spécifique de la bactérie à des caractères aspécifiques de la plante, soit par une adaptation aspécifique de la bactérie à des propriétés spécifiques d'un génotype de plante. Ainsi, nous formulons l'hypothèse que ces adaptations se traduisent par la régulation de nombreux gènes indépendamment de leur implication directe dans l'effet phytobénéfique. Ces régulations pourraient dépendre de la combinaison souche bactérienne/génotype de plante considérée. L'objectif de ce travail est de mettre en évidence les gènes impliqués dans l'adaptation des partenaires l'un à l'autre et ceux impliqués dans la spécificité, au cours de la coopération PGPR-plantes. Pour cela, nous avons choisi comme modèle d'étude l'interaction entre *Azospirillum lipoferum* 4B et les céréales (blé, maïs, riz cultivars Cigalon et Nipponbare) avec un accent porté sur l'expression des gènes des deux partenaires de la coopération *A. lipoferum* 4B-riz. Les résultats de transcriptomique sur puce soulignent l'importance des mécanismes de réponse au stress oxydatif au cours de l'adaptation des partenaires l'un à l'autre ainsi que la mise en place de réseaux de régulation complexes. De nombreux gènes présentent un profil d'expression qui dépend de la combinaison souche/cultivar, suggérant que des mécanismes évolutifs ont conduit à une interaction préférentielle entre une souche et son cultivar d'origine.

Mots clés : *Azospirillum*, Blé, Coopération, Maïs, Phytostimulation, Rhizosphère, Riz, Spécificité d'hôte, Transcriptomique.

Abstract

Host specificity is a fundamental concept in understanding evolutionary processes leading to intimate interactions between bacteria and plants. In the case of Plant Growth-Promoting Rhizobacteria (PGPR) specificity appears to be controlled either by a strain-specific bacterial adaptation to non-specific traits of the host plant or by non-specific bacterial adaptation to genotype-specific properties of the host plant. Thus, we hypothesize that these adaptations result in the regulation of a large number of genes, independently of their direct involvement in phytostimulation. These regulations may depend on the bacterial strain / plant genotype combination. This work aims at identifying genes involved in reciprocal adaptation of partners and those involved in host specificity in PGPR-plant cooperation. As a model, we studied the interaction between *Azospirillum lipoferum* 4B and cereals (wheat, corn, rice cultivars Nipponbare and Cigalon), with an emphasis on gene expression of both partners during *A. lipoferum* 4B-rice cooperation. Microarray transcriptomic results highlight the importance of mechanisms implicated in response to oxidative stress as well as a tight adjustment of regulatory networks during the adaptation of both partners to each other. Many genes display expression profile that depends on the strain/cultivar combination, suggesting that evolutionary processes have led to a preferential interaction between a strain and its original cultivar.

Keywords: *Azospirillum*, Cooperation, Host Specificity, Maize, Phytostimulation, Rhizosphere, Rice, Transcriptomic, Wheat.

Vivre,

Apprendre,

Comprendre ses erreurs,

Et assumer ses choix.

bd

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Liste des figures

Liste des tableaux

Liste des abréviations

ABA : Abscisic acid

ACC : 1-aminocyclopropane-1-carboxylic acid

AHL : N-acyl homoserine lactones

c-di-GMP : bis-(3',5')-cyclic-dimeric-guanosine monophosphate

CRP : Cyclic AMP receptor protein

DAPG : 2,4-diacetylphloroglucinol

DKP : Diketopiperazines

EPS : Exopolysaccharides

ETI : Effector-Triggered Immunity

FC : Fold Change

FNR : Fumarate-nitrate reduction regulatory protein

IAA: Indole-3-acetic acid

ISR : Induced Systemic Resistance

JA : Jasmonate

LRP : Leucine-responsive regulatory protein

MAMPs : Microbe-associated molecular patterns

MDR : Multi-Drug Resistance

MeSA : Methyl Salicylate

NO : Nitric Oxide

PAMPs : Pathogen-associated molecular patterns

PGPR : Plant growth-promoting rhizobacteria

PLT : Pyoluteorin

PTI : PAMPs-Triggered Immunity

PR : Pathogenesis-related

QS : Quorum-sensing

RND : Resistance-Nodulation-Division

ROS : Reactive oxygen Species

SA : Salicylic Acid

SAR : Systemic Acquired Resistance

SMR : Small Multidrug Resistance

Trp : Tryptophan

VOC : Volatile organic compounds

WGA : Wheat Germ Agglutinin

Sommaire

Chapitre III : Expression des gènes du riz en réponse à l'interaction avec *Azospirillum.*

Introduction Générale

Les symbioses jouent un rôle essentiel dans le fonctionnement des écosystèmes et l'évolution des êtres vivants. D'après la théorie endosymbiotique (**Figure 1**), la symbiose entre une cellule eucaryote primitive et une cellule procaryote hébergée au sein même de son cytoplasme aurait abouti à la formation des mitochondries et des chloroplastes (Margulis 1970). De même, la colonisation du milieu terrestre par les Eucaryotes aurait été facilitée par la symbiose entre un organisme photosynthétique et un organisme fongique (Heckman *et al.* 2001).

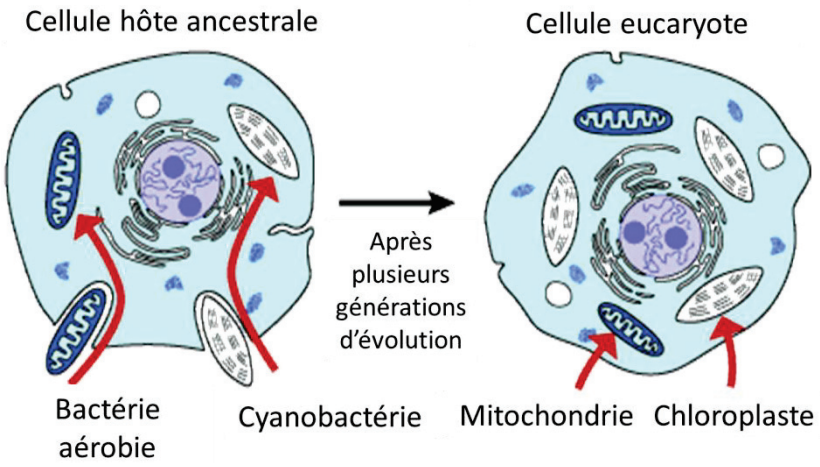

Figure 1 : Théorie endosymbiotique de l'Evolution
(source : http://evolution.berkeley.edu/evolibrary/article/history_24)

A l'origine, le terme symbiose désignait une association délétère ou bénéfique de différents organismes (de Bary 1879) ; nous l'utiliserons ici au sens d'une interaction intime et durable entre deux êtres vivants qui en partagent les coûts et les bénéfices. Les symbioses mutualistes (mutualisme) sont considérées comme obligatoires car indispensables à la survie de chacun des partenaires (Odum 1971). Elles aboutissent à une spécialisation importante, menant généralement à la formation d'une structure spécifiquement dédiée à l'interaction : c'est par exemple le cas des symbioses *Rhizobium*-Légumineuses et *Vibrio*-Calamar (**Figure 2 A,B et C,D**) (Masson-Boivin *et al.* 2009 ; Nyholm et McFall-Ngai 2004). Les symbioses associatives (coopérations) sont quant à elles considérées comme facultatives, car les bénéfices apportés ne sont pas indispensables à la survie des organismes qu'elles impliquent (Moënne-Loccoz *et al.* 2011). Malgré les modifications physiologiques induites par les coopérations, aucune structure spécifique n'est formée : c'est par

exemple le cas des interactions *Azospirillum*-Plantes et *Lactobacillus*-Vertébrés (**Figure 2 E,F et G,H**) (Bashan et de Bashan 2010 ; Dethlefsen *et al.* 2007 ; Frese *et al.* 2011).

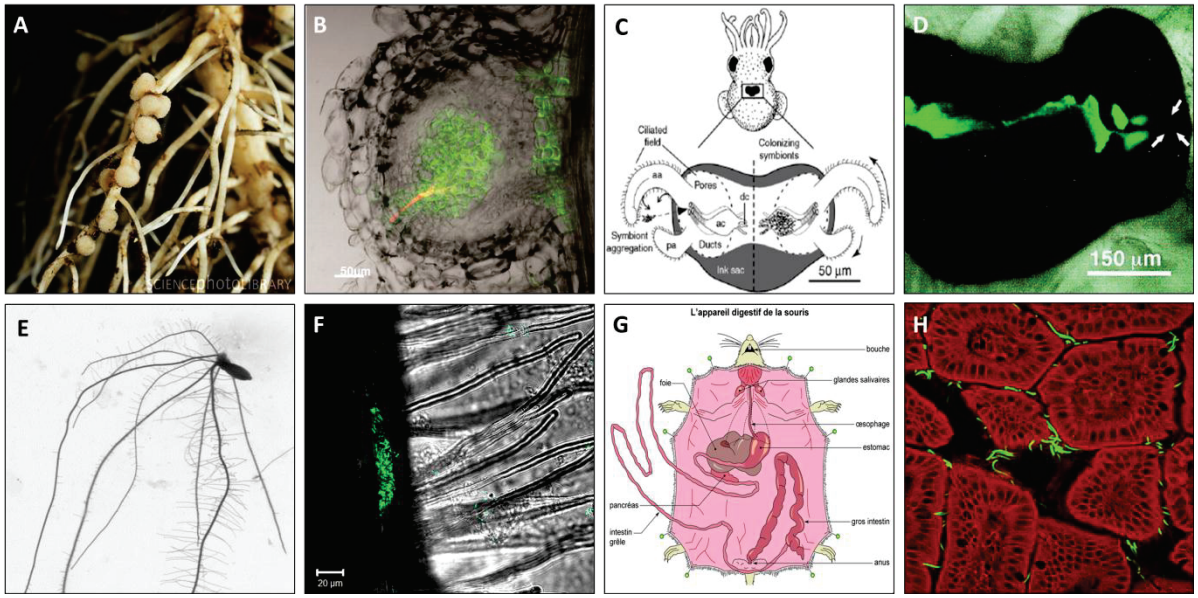

Figure 2 : Exemples de symbioses mutualistes et de symbioses associatives.
A,B,C,D, Symbiose mutualiste. E,F,G,H, Symbiose associative. A,E, Photographies de systèmes racinaires. C,G, Schémas. B,D,F,H, Images de microscopie à fluorescence montrant des bactéries produisant l'EGFP (vert). A, Nodosités sur des racines de fève (source : www.sciencephoto.com). B, Coupe transversale d'une nodosité (source : Svistoonoff *et al.* 2004). C, Organe lumineux du calamar (source : Visick et Ruby 2006). D, Population de *Vibrio fisheri* dans l'organe lumineux du calamar (source : Nyholm *et al.* 2009). E, Racine de riz colonisée par *Azospirillum* (source : ce travail). F, Population d'*Azospirillum lipoferum* 4B sur la surface racinaire du riz (source : ce travail). G, Appareil digestif de la souris (source : www.assistancescolaire.com). H, Population bactérienne (vert) à la surface des cellules intestinales (rouge) (source : « My microbes » dans http://news.harvard.edu/).

Bien qu'elles soient facultatives, les coopérations ont un rôle central dans les écosystèmes, notamment au niveau de la rhizosphère des plantes. En effet, ce volume de sol au contact et sous l'influence directe des racines (Hiltner 1904), représente un environnement riche en composés organiques qui favorisent la croissance et l'activité microbiennes (Bais *et al.* 2006). En retour, certaines bactéries rhizosphériques dites PGPR (*Plant Growth-Promoting Rhizobacteria*) améliorent la croissance et la santé des plantes (Lugtenberg et Kamilova 2009 ; Vessey 2003). De par leur intérêt agronomique majeur, notamment pour remplacer des intrants chimiques dans le contexte d'une agriculture durable, les PGPR ont été largement étudiées ces cinquante dernières années. Elles sont réparties en trois catégories (**Figure 3**): (i) les PGPR phytostimulatrices stimulent directement la

croissance des plantes *via* une modification de leur balance hormonale et une amélioration de leur nutrition hydrominérale (Lugtenberg et Kamilova 2009) ; (ii) les PGPR phytoprotectrices protègent la plante contre les pathogènes par la production de composés antimicrobiens ou par l'induction de la résistance systémique de la plante (Compant *et al.* 2005 ; Lugtenberg et Kamilova 2009) ; enfin, (iii) les PGPR auxiliaires (*helper*) favorisent la mise en place d'autres symbioses plantes-microorganismes, notamment avec les champignons mycorhiziens ou les bactéries nodulantes (Frey-Klett *et al.* 2007 ; Fox *et al.* 2011 ; Dardanelli *et al.* 2008).

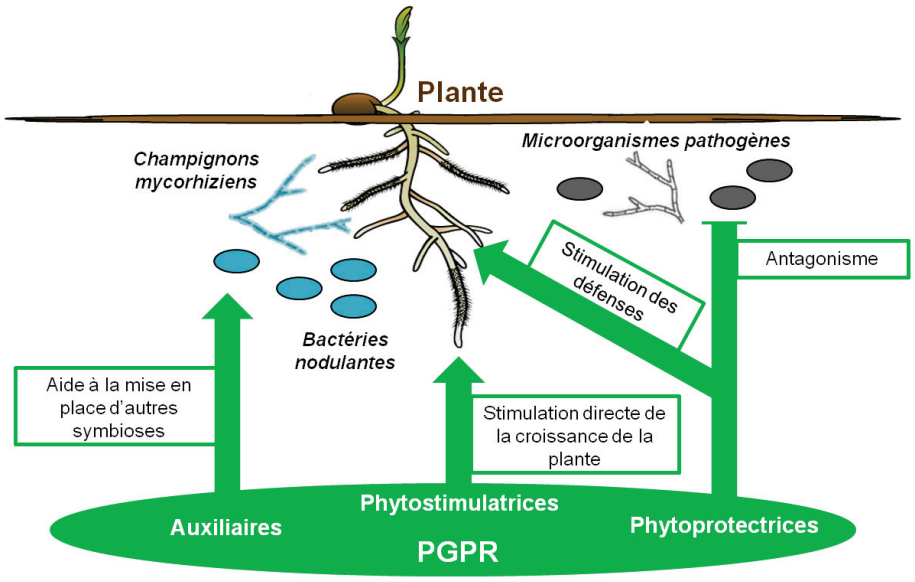

Figure 3 : Principaux modes d'action des PGPR

Dans le cas des PGPR phytostimulatrices, de nombreuses études sont réalisées d'un point de vue anthropocentrique, considérant principalement les effets bénéfiques pour la plante en termes de croissance ou de rendement ainsi que les mécanismes biologiques directement impliqués dans l'effet phytobénéfique (Fuentes-Ramirez et Caballero-Mellado 2005 ; Hayat *et al.* 2010 ; Lugtenberg et Kamilova 2009 ; Veresoglou et Menexes 2010 ; **Annexe I**). Parmi ces études, plusieurs mettent en évidence que les effets des PGPR phytostimulatrices dépendent de la souche bactérienne et du génotype de plante (Chanway *et al.* 1988 ; Gunarto *et al.* 1999 ; Gyaneshwar *et al.* 2002 ; Mehnaz et Lazarovits 2006 ; Moutia *et al.* 2010; Pedraza *et al.* 2010 ; Walker *et al.* 2010). En revanche, les mécanismes d'adaptation

des partenaires l'un à l'autre, et ceux qui régissent la spécificité d'hôte restent peu étudiés.

Dans ce contexte, le **Chapitre I** de ce travail est une synthèse des connaissances actuelles concernant : l'impact des signaux rhizosphériques sur le fonctionnement de la coopération PGPR-plantes (**Partie 1**) ; les mécanismes potentiellement impliqués dans la spécificité d'hôte au cours de la coopération, avec un accent porté sur les PGPR phytostimulatrices (**Partie 2**) ; et la réponse immunitaire de la plante et les mécanismes induits par la perception des PGPR. Chez les PGPR phytostimulatrices, la spécificité d'hôte semble régie soit par une adaptation souche-spécifique de la bactérie à des caractères aspécifiques de la plante, soit par une adaptation aspécifique de la bactérie à des propriétés spécifiques d'un génotype de plante (**Chapitre I).** Ainsi, la capacité à coloniser et par conséquent stimuler la croissance d'un groupe restreint de végétaux pourrait découler d'un contrôle fin des propriétés phytobénéfiques des microorganismes (Smith et Goodman, 1999 ; Berg et Smalla 2009 ; Kloepper 1996).

Nous formulons ici l'hypothèse que l'adaptation des partenaires l'un à l'autre se traduit par la régulation d'un grand nombre de gènes indépendamment de leur implication directe dans l'effet phytobénéfique. Ces régulations pourraient dépendre de la combinaison souche bactérienne/génotype de plante considérée.

L'objectif de ce travail de thèse est de mettre en évidence les déterminants génétiques impliqués dans l'adaptation des partenaires l'un à l'autre et ceux impliqués dans la spécificité d'hôte, au cours de la coopération entre les PGPR phytostimulatrices et les plantes. Pour cela, nous avons choisi comme modèle d'étude la coopération entre les alpha-protéobactéries du genre *Azospirillum* et les céréales. L'expression des gènes du partenaire végétal (riz) et celle du partenaire bactérien (*Azospirillum lipoferum* 4B) ont été analysées à l'aide de puces transcriptomiques, sept jours après inoculation.

L'expression des gènes de la souche *A. lipoferum* 4B a été étudiée au cours de la coopération avec le riz, le maïs et le blé. Ce travail, décrit dans le **Chapitre II** se

décompose en deux parties. La **Partie 1** évalue l'impact de la variabilité intraspécifique de la plante hôte, sur le transcriptome bactérien. Ainsi, *A. lipoferum* 4B a été inoculée sur deux cultivars de riz (*Oryza sativa* L. *japonica* cv. Cigalon, cultivar à partir duquel elle a été isolée, et cv. Nipponbare). De la même manière, la **Partie 2** considère cet impact à l'échelle interspécifique en comparant l'expression des gènes bactériens après sept jours de coopération avec le riz (*Oryza sativa* L. *japonica* cv. Cigalon), le maïs (*Zea mays* L. cv. PR37Y15) et le blé (*Triticum aestivum* L. cv. Soissons).

De précédentes études sur le métabolome et la croissance de deux cultivars de riz (cv. Cigalon et du cv. Nipponbare) en réponse à l'inoculation par *Azospirillum* (*A. lipoferum* 4B isolée du cv. Cigalon et *Azospirillum sp*. B510 isolée du cv. Nipponbare) ont montré la mise en place d'une interaction particulière entre une souche et son cultivar d'origine (Chamam *et al.* sous presse). Afin de mettre en évidence les déterminants génétiques potentiellement impliqués dans cette réponse particulière, le travail décrit dans le **Chapitre III** vise à déterminer l'impact des souches *A. lipoferum* 4B et *Azospirillum sp*. B510 sur l'expression des gènes des cultivars Cigalon et Nipponbare.

Enfin, la discussion générale propose une synthèse des résultats de transcriptomique obtenus pour les deux partenaires, dans le but de mieux comprendre les mécanismes impliqués dans la spécificité d'hôte et de proposer des perspectives d'études.

Chapitre I :

Synthèse bibliographique

Partie 1 :

Contrôle de la coopération entre les bactéries phytobénéfiques et les plantes *via* les signaux rhizosphériques

Benoît Drogue, Emeline Combes-Meynet, Yvan Moënne-Loccoz, Florence Wisniewski-Dyé et Claire Prigent-Combaret

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Molecular Microbial Ecology of the Rhizosphere,

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Control of the cooperation between plant growth-promoting rhizobacteria and crops by rhizosphere signals

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Running title: Signal effects of plant and PGPR-derived molecules within the rhizosphere.

Keywords: rhizosphere signals, PGPR, primary and secondary metabolites, phytohormones, antimicrobial compounds, quorum sensing, plant beneficial genes, spatial population distribution.

Abstract

In the rhizosphere, an extensive communication takes place between plants and root-associated microorganisms, and involving an astonishing diversity of compounds released by plants (as root exudates) and rhizobacteria. These organic compounds may play a role as nutrients, toxins and/or signals, depending on the microbial partner as well as compound concentration. Therefore, they can be a major driver determining the assembly and functioning of rhizosphere communities. Until now, chemical signalling has been mostly studied during interaction between plants and pathogens, but recent work evidenced the involvement of several chemical signals in controlling the associative symbiosis between plant growth-promoting rhizobacteria (PGPR) and roots. In this review, recent findings about the identification of signals involved in the interaction of plants with PGPR, and of PGPR with each others, are presented. First, the focus is put on primary and secondary metabolites produced by plants, which may control the expression of PGPR genes, particularly those involved in plant-beneficial properties. Second, the role of signals released by PGPR, such as phytohormones, volatile organic compounds (VOC), N-acyl homoserine lactones (AHL) or antimicrobial compounds, on modulation of plant growth and health, is considered. Third, signal molecules involved in cross-talk between PGPR are examined. Finally, the spatial distribution of PGPR populations on roots is emphasized as a key factor influencing PGPR's relationships with other rhizosphere-inhabiting partners.

I.1.1. Introduction

The rhizosphere is a very active microbial habitat where extensive communication occurs between plant roots and rhizobacteria, and between rhizobacteria themselves (Ortíz-Castro *et al.* 2009). Molecules exuded by plant roots are mostly used as nutrients allowing rhizobacteria, which are able to metabolize them, to colonize plant roots and to survive in the rhizosphere. However, many compounds found in this habitat, released either by plants or by rhizobacteria, may also act as signals.

Signals correspond to low molecular weight and diffusible compounds that are emitted by an organism, perceived by another and that elicit, at low concentrations ranging from nanomolar to micromolar, a specific response in the latter through a signal-transducing cascade (Bais *et al.* 2006; Hirsch *et al.* 2003). Two component systems correspond to a widespread mechanism involved in signal-transduction in rhizobacteria, an issue recently reviewed by Faure *et al.* (2009).

The rhizosphere contains a myriad of molecule types including primary metabolites like amino acids and sugars, and secondary metabolites like indoles, N-acyl homoserine lactones, or phloroglucinols whose concentrations will vary in the rhizosphere habitat according to the distance from their emission point. Thus, some of these molecules in definite zones of the rhizosphere will actually act as signals.

This chapter will focus on rhizosphere signals, produced by plants or Plant Growth-Promoting Rhizobacteria (PGPR), that affect the ecology and gene expression of PGPR. PGPR are beneficial rhizobacteria colonizing the plant root surface and stimulating the growth and health of a wide range of economically important crops through various direct and indirect mechanisms. Rhizosphere signalling has been the focus of several recent reviews but with no specific highlight on PGPR (Hirsch *et al.* 2003; Faure *et al.* 2009; Ortíz-Castro *et al.* 2009). We will describe first, signal compounds produced by plants that affect PGPR gene expression, with a focus on genes especially involved in plant-beneficial properties, second, signal molecules produced by PGPR that affect the growth of the host plant,

and finally signal molecules produced by PGPR that affect gene expression in other PGPR.

I.1.2. Plant signals with effects on plant beneficial properties of PGPR

Plant roots release in the soil a wide range of both low and high molecular weight compounds like sugars, organic acids, phenols, vitamins, amino acids, proteins, and mucilages (Badri and Vivanco 2009; Bais *et al.* 2006). These molecules correspond to primary and secondary metabolites that can be used both as nutrients or signals by rhizobacterial populations. Roots also secrete volatile organic compounds (VOC) that are well known as mediators of plant-plant (allelopathy) and plant-invertebrate communications, but whose effect on rhizobacteria has been under-investigated (Wenke *et al.* 2010). The qualitative and quantitative composition of root exudates varies depending on the plant genotypes, plant phenology, and environmental factors (Bais *et al.* 2006), but overall, it is estimated that up to 40% of photosynthetically fixed carbon could be transferred to the rhizosphere (Lynch and Whipps 1990).

Several studies have reported that seed or plant exudates induce major changes of gene expression in PGPR. Genes with enhanced expression include genes encoding regulator systems, genes involved in root colonization and plant growth promotion in *Azospirillum brasilense* Sp245 (Pothier *et al.* 2007), in chemotaxis and type III secretion in *Pseudomonas aeruginosa* PAO1 (Mark *et al.* 2005).

I.1.2.1. Regulation of bacterial gene expression by plant primary metabolites

Primary metabolites such as amino acids and sugars can control gene expression in PGPR independently of trophic effects (**Table 1**).

Table 1. Signal effect of plant-derived compounds during the cooperation between plants and PGPR.

Compounds	PGPR strains	Regulated functions	References
Primary metabolites			
D-galactose, L-arabinose, D-fructose, malate, succinate, fumarate	*Azospirillum brasilense*, *Bacillus subtilis*	Chemotaxis, biofilm formation, rhizocompetence	Van Bastelaere *et al.* 1999; Kamilova *et al.* 2006; Rudrappa *et al.* 2008
Glucose, glycerol, sucrose, fructose, mannitol, IAA	*Pseudomonas protegens*, *Pseudomonas fluorescens*	Production of antimicrobial compounds such as DAPG, PLT, or pyrrolnitrin	Duffy and Défago 1999; Shanahan *et al.* 1992; de Werra *et al.* 2011
Tryptophan, tyrosine, phenylalanine, IAA, phenylacetic acid, naphthalene acetic acid, pyridoxine, nicotinic acid	*A. brasilense*, *Bacillus amyloliquefaciens*, *Enterobacter cloacae*, *P. fluorescens*	IAA biosynthesis	Bashan and de-Bashan, 2010; Idris *et al.* 2007; Kamilova *et al.* 2006; Ona *et al.* 2005; Rothballer *et al.* 2005; Ryu and Patten 2008; Somers *et al.* 2005; Vande Broek *et al.* 1999
Leucine, glucose	*Azospirillum lipoferum*, *Pseudomonas putida*	Biosynthesis of ACC deaminase	Glick *et al.* 2007; Prigent-Combaret *et al.* 2008; Grichko and Glick 2000
Secondary metabolites			
Naringenin, daidzein, myricetin	*A. brasilense*, *Azorhizobium caulinodans*	Root colonization	Jain and Gupta 2003; Webster *et al.* 1998
AHL mimics	*P. protegens*, *P. fluorescens*	Bacterial cell communication, plant beneficial properties	Subramoni *et al.* 2011
Salicylic acid, catechin, umbelliferone, quinolinic acid, 8-quinolinol, jasmonate, methyl-jasmonate, coumaric acid, cinnamic acid, benzaldehyde, acetosyringone, acetovanillone, resorcinol	*P. protegens*	Production of DAPG and PLT antimicrobial compounds	Schnider-Keel *et al.* 2000; Baehler *et al.* 2005; de Werra *et al.* 2011

DAPG: 2,4-diacetylphloroglucinol. IAA: indole-3-acetic acid. PLT: pyoluteorin.

Tryptophan (Trp) is a biosynthetic precursor of indoleacetic acid, an auxin widely produced by PGPR; however, some less-characterized Trp-independent biosynthetic pathways exist (Spaepen *et al.* 2007a). Bacterial auxin biosynthesis depends on the Trp content inside bacterial cells, which results from bacterial production or uptake from the rhizosphere. The Trp content in root exudates is extremely variable according to the plant species (Kamilova *et al.* 2006) and to root

zones (Jaeger *et al.* 1999). Besides a precursor effect, Trp has been shown to modulate the transcription of the *ipdC/ppdC* gene (encoding the indole-3-pyruvate decarboxylase activity involved in IAA biosynthesis) in *A. brasilense* and in *Enterobacter cloacae*; thereby, Trp can affect the level of auxin produced by those PGPR and consequently their plant growth-promoting effects on the host plant (Ona *et al.* 2005; Rothballer *et al.* 2005; Ryu and Patten 2008; Spaepen *et al.* 2008).

Other amino-acids such as tyrosine and phenylalanine also up-regulate the expression of *ppdC* (Rothballer *et al.* 2005; Ryu and Patten 2008). In *E. cloacae*, aromatic acid-dependent expression of *ipdC* involves the TyR transcriptional regulator (Ryu and Patten 2008). Leucine is a branched chain amino acid that directly affects the transcription of leucine-responsive regulatory protein (LRP) dependent genes such as *acdS*, which encodes 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. This activity is involved in the deamination of ACC, the immediate precursor of plant ethylene. This leads to a decrease of ethylene production in plants. Because ethylene inhibits root growth and may be produced in too large amounts during plant stress response, bacterial ACC deamination can enhance both root system development and plant stress tolerance (Penrose and Glick 2003; Cheng *et al.* 2007). The binding of leucine to the LRP-like transcriptional regulator AcdR affects its oligomerization state. Consequently, AcdR becomes unable to bind to the fumarate-nitrate reduction regulatory protein (FNR) and cyclic AMP receptor protein (CRP) required for transcription of acdS in anaerobic and aerobic conditions, respectively and, in such a way, to activate the transcription of *acdS* (Glick *et al.* 2007; Grichko and Glick 2000; Prigent-Combaret *et al.* 2008).

Many root-exuded sugars and dicarboxylic acids such as malate, succinate and fumarate act as chemoattractants (Kamilova *et al.* 2006) and activate the expression of genes involved in bacterial chemotaxis. For example, in *A. brasilense*, D-galactose, L-arabinose and D-fucose induce the expression of the *sbpA* gene encoding the sugar-binding protein A (Van Bastelaere *et al.* 1999). Sugars regulate the expression of many other genes involved in the interaction of PGPR with plants, such as glucose that slightly down-regulates the transcription (CRP regulation) of *acdS* in *Azospirillum lipoferum* 4B (Prigent-Combaret *et al.* 2008). Root-secreted

malic acid up-regulates the transcription of *yqxM*, a gene involved in biofilm formation, of the beneficial soil bacterium *Bacillus subtilis* FB17 and accordingly its root colonization abilities (Rudrappa *et al.* 2008). Finally, many organic acids modulate the expression of plant-beneficial genes such as phenylacetic acid acting on *ppdC* in *A. brasilense Sp245* (Somers *et al.* 2005), or gluconic acid acting on *phlA* and *pltA* in *P. protegens* (formerly *P. fluorescens*) CHA0 (de Werra *et al.* 2009).

I.1.2.2. Regulation of rhizobacterial gene expression by plant secondary metabolites

In addition to primary metabolites, roots secrete a large range of secondary metabolites including salicylic acid, flavonols and indole compounds (Badri and Vivanco 2009). These compounds can act as signals on rhizobacteria and influence the success of beneficial PGPR-plant interactions, from the early stage to the established cooperation, improving root colonization and regulating expression of plant-beneficial properties (**Table 1**).

While they are mostly studied for their implication in the initiation of the *Rhizobium-Fabaceae* symbiosis, flavonoids may represent a major signal for root colonization by PGPR. Wheat root colonization by the diazotrophic bacterium *Azospirillum brasilense* Sp245 is stimulated in the presence of flavonoids (Webster *et al.* 1998). Indeed, addition of 100 µM of daidzein, myricetin, or naringenin to the plant growth medium significantly increases (2 to 3 times) the percentage of lateral root cracks colonized per plant. More recently, similar results have been reported in rice (Jain and Gupta 2003).

The expression of biocontrol-relevant genes from plant-beneficial pseudomonads are modulated by a wide range of plant signals. *P. protegens* CHA0 produces the two well-characterized antifungal compounds, 2,4-diacetylphloroglucinol (DAPG) (Keel *et al.* 1992) and pyoluteorin (PLT) (Maurhofer *et al.* 1994). The balance between these two antifungal compounds is finely regulated in strain CHA0 in response to plant-derived factors (de Werra *et al.* 2011). In a screening of 63 plant-derived compounds (flavonoids, phenolic acids, phytohormones, etc) for their ability to modulate the expression of *phlA* and *pltA* in

CHA0, two genes involved in DAPG and PLT biosynthesis respectively, it appeared that numerous plant compounds could regulate these genes. However no particular chemical structure was identified to specifically induce or repress *phlA* or *pltA* gene expression (de Werra *et al.* 2011). Well-known plant signals involved in plant defense like salicylate (Schnider-Keel *et al.* 2000), jasmonate, and methyl jasmonate, all slightly reduced *phlA* gene expression, whereas the plant hormone indole-3-acetic acid induces its expression. None of these compounds has an effect on the expression of *pltA*.

Phytohormones and their precursors are implicated in plant growth and defense response; but as these compounds are also exuded in the rhizosphere, their presence can modify the expression of the plant-beneficial properties of PGPR. For example, addition of ACC significantly activates *acdS* transcription in the plant growth-promoting strain *Pseudomonas putida* UW4 (Grichko and Glick 2000). In the phytostimulator *Azospirillum lipoferum* 4B, the level of *acdS* transcription and ACC deaminase activity are reduced 4.4 fold and 2.2 fold respectively without ACC (Prigent-Combaret *et al.* 2008).

The ability of *Azospirillum* to synthesize and secrete phytohormones such as indole-3-acetic acid (IAA) is considered as a key property contributing to plant growth promotion (Fibach-Paldi *et al.* 2011). Interestingly, *ipdC*/*ppdC* transcription is positively regulated by IAA and other auxins such as naphataleneacetic acid and phenylacetic acid (Somers *et al.* 2005; Spaepen *et al.* 2007b; Vande Broek *et al.* 1999). On a larger scale, a microarray transcriptomic analysis of *Azospirillum brasilense* Sp245 wild-type and *ipdC* mutant, with or without exogenous IAA, revealed that IAA is a signalling molecule in *A. brasilense* (Van Puyvelde *et al.* 2011). Indeed, 160 genes were found to be regulated regardless of the source of IAA, including 34 genes of unknown function (the part of which could be specific for the plant/*A. brasilense* association), signal transduction genes, metabolism related systems, bacterial nitrate reducing system (*nap* genes), tripartite ATP-independent periplasmic transporters and components of the type VI secretion system. These results show that IAA modifies the regulatory machinery and alters the repertory of surface and transporter proteins in *A. brasilense* Sp245, suggesting that IAA is a

means of communication between *Azospirillum* and the host plant (Van Puyvelde *et al.* 2011). Moreover, it has also been shown that IAA induces the expression of genes in *E. coli* related to survival under stress conditions (Bianco *et al.* 2006). Thus, IAA seems to act as a signalling molecule not only involved in communication with plants, but also as a communication molecule between bacteria and other microorganisms.

Among plant secondary metabolites that act as signal in bacteria, the least investigated are bioactive components that interfere with PGPR quorum-sensing (QS). Initially, exudates from pea, vetch, soybean and rice have been shown to contain compounds that mimic bacterial N-acyl homoserine lactones (AHL) and stimulate or inhibit some QS systems (Gao *et al.* 2003; Teplitski *et al.* 2000); however, the chemical nature of these AHL mimics remains unknown. Many other plant species have been screened for the presence of compounds interfering with QS-regulated phenotypes of bacterial pathogens; in this context, some flavonoids were recently shown to disrupt QS-regulated phenotypes in the opportunistic pathogen *Pseudomonas aeruginosa* (Vandeputte *et al.* 2009, 2010). As key physiological properties involved in PGPR beneficial effects are known to be regulated by QS, plant AHL mimics may also interfere with QS regulation in PGPR. Moreover, some biocontrol *Pseudomonas fluorescens* strains lack a *luxI*-type gene encoding the AHL synthase but contain a LuxR-like receptor able to sense plant molecule(s) and modulate expression of genes involved in biocontrol properties (Subramoni *et al.* 2011). Thus, plant AHL mimics can strongly influence the success of PGPR-plant interactions.

I.1.3. Bacterial signals with direct effects on plant growth

PGPR can stimulate the growth of plants through the production of a wide diversity of signal molecules like phytohormones, VOC, phloroglucinols or AHL. Those compounds act, at concentrations ranging from nanomolar to micromolar, either as direct plant growth stimulatory factors or both as plant growth regulators and plant stress protecting factors.

I.1.3.1. Plant growth regulators produced by PGPR

Production of phytohormones by PGPR leads to distinct effects on plant development and root architecture summarized in **Table 2**.

Table 2. Effects of PGPR-derived compounds on the growth of plants.

Compounds	PGPR strains	Plant effects	References
With plant growth-promoting properties			
IAA	*Azospirillum brasilense, Bacillus amyloliquefaciens, Enterobacter cloacae, Pantoea agglomerans, Pseudomonas putida*	Proliferation of root hair, formation of lateral roots, inhibition of root length	Dobbelaere *et al.* 1999; Idris *et al.* 2007; Patten and Glick 2002; Spaepen *et al.* 2007a
Cytokinin	*Arthrobacter giacomelloi, A. brasilense, Bradyrhizobium japonicum, Pseudomonas fluorescens, Paenibacillus polymyxa*	Proliferation of root hairs, inhibition of lateral root formation, inhibition of primary root elongation	Cacciari *et al.* 1989; Cassán *et al.* 2009; de García Salamone *et al.* 2001; Perrig *et al.* 2007; Timmusk *et al.* 1999
Gibberellin	*Azospirillum lipoferum, Azotobacter* spp., *Bacillus pumilus, Bacillus licheniformis, Br. japonicum, Herbaspirillum seropedicae, Gluconobacter diazotrophicus*	Promotion of root elongation, extension of lateral roots	Bottini *et al.* 2004; Cassán *et al.* 2009; Gutiérrez-Mañero *et al.* 2001; Perrig *et al.* 2007
With plant growth-promoting and stress alleviating properties			
Abscissic acid	*A. lipoferum, A. brasilense*	Stress alleviation	Cohen *et al.* 2008, 2009
Ethylene	*A. brasilense*	Inhibition of root elongation, induction of systemic resistance (ISR), plant defense pathways	Perrig *et al.* 2007; Ribaudo *et al.* 2006
Nitric oxide	*A. brasilense*	Formation of lateral and adventitious roots, plant defense pathways	Creus *et al.* 2005; Molina-Favero *et al.* 2008
Volatile organic compounds	*B. subtilis, B. amyloliquefaciens, E. cloacae*	Control of root morphogenesis and induction of ISR	Kai *et al.* 2009 ; Ryu *et al.* 2003
AHL	*P. putida,* and other PGPR	Promotion of lateral root development, root hair density and growth, induction of ISR	Ortiz-Castro *et al.* 2008; Schuhegger *et al.* 2006
DAPG	*P. fluorescens, Pseudomonas protegens*	Induction of ISR, stimulation of root exudation, promotion of root branching	Bakker *et al.* 2007; Brazelton *et al.* 2008; Iavicoli *et al.* 2003; Phillips *et al.* 2004

AHL: *N*-acyl-homoserine lactone. DAPG: 2,4-diacetylphloroglucinol. IAA: indole-3-acetic acid.

IAA is a common auxin produced by rhizobacteria (Ahmad *et al.* 2008; Khalid *et al.* 2004; Patten and Glick 1996; Spaepen *et al.* 2007a). It is usually synthesized from tryptophan which is found in root exudates at different concentrations according to plant genotype (Kamilova *et al.* 2006). Several IAA biosynthetic pathways, classified according to the metabolic intermediates have been described (Patten and Glick 1996; Spaepen *et al.* 2007a).

However, only a few specific genes and enzymes involved in IAA biosynthesis have been characterized so far, and in a quite restricted number of PGPR including *Azospirillum brasilense, Bacillus amyloliquefaciens, Enterobacter cloacae, Pantoea agglomerans* and *Pseudomonas putida* species (Dobbelaere *et al.* 1999; Idris *et al.* 2007; Patten and Glick 2002; Spaepen *et al.* 2007a). The production of IAA by PGPR enhances root branching, resulting in a greater surface of the root system. Thus, plant roots can explore a wider soil volume and thereby improve the mineral and aqueous nutrition of the plant; bacteria, in return, can colonize an increased root surface and benefit from potentially enhanced root exudation. Indeed, some but not all studies have shown that the ability to colonize plant roots is reduced for mutants unable to produce IAA (Brandl and Lindow 1998; Patten and Glick 2002; Suzuki *et al.* 2003). The impact of exogenous auxin on plant development differs according to the amount of IAA and the sensitivity of the host plant to changes in exogenous IAA concentrations (Dobbelaere *et al.* 1999; Perrig *et al.* 2007; Remans *et al.* 2008; Spaepen *et al.* 2008). Besides IAA production, *Azospirillum* in particular seems to produce several other key auxin-type molecules like indole butyric acid (Fallik *et al.* 1989), or phenyl acetate (Somers *et al.* 2005).

Cytokinin production (especially zeatin) has been documented in various PGPR like *Arthrobacter giacomelloi, Azospirillum brasilense, Bradyrhizobium japonicum, Pseudomonas fluorescens* and *Paenibacillus polymyxa* (Cacciari *et al.* 1989; Cassán *et al.* 2009; de García Salamone *et al.* 2001; Perrig *et al.* 2007; Timmusk *et al.* 1999). However, the real contribution of cytokinin production by PGPR to plant growth promotion is rather speculative so far, because bacterial genes involved in putative cytokinin bacterial biosynthetic pathways were identified by

comparative genomics and their role has not been validated by functional studies (Frébort *et al.* 2011).

I.1.3.2. PGPR factors with dual plant growth-promoting and stress alleviating properties

a. Phytohormones

Bacterial production of abscisic acid (ABA) has been less studied. The ability of bacterial ABA to promote plant growth has been documented in *A. lipoferum* and *A. brasilense*, but a positive effect has been only observed in water-stressed plants (Cohen *et al.* 2008, 2009).

Another key phytohormone involved in plant stress alleviation by PGPR is ethylene. Ethylene is required for the induction of systemic resistance (ISR) in plants during associative and symbiotic plant-bacteria interactions and, at higher concentrations, is involved in plant defense pathways induced in response to pathogen infection (Broekaert *et al.* 2006; Glick *et al.* 2007). Certain PGPR such as *A. brasilense* have been shown to produce small amounts of ethylene from methionine as a precursor (Perrig *et al.* 2007), and this ability seems to promote root hair development in tomato plants (Ribaudo *et al.* 2006). However, characterization of the bacterial biosynthetic pathway and genetic determinants has to be performed in order to confirm the role of ethylene in the growth promoting effect of PGPR in presence or absence of stress.

Increasing evidence indicates that NO is a key signalling molecule involved in a wide range of effects on plants (Creus *et al.* 2005; Molina-Favero *et al.* 2008). NO was shown to play an important role in auxin-regulated signalling networks, influencing root organogenesis (Pagnussat *et al.* 2002). Some plant-associated bacteria such as *A. brasilense* (strain Sp245) are able to produce NO due to the activity of nitrite reductases (Creus *et al.* 2005; Pothier *et al.* 2007; Steendhoudt *et al.* 2001). NO is an intermediate in the denitrification pathway. Although denitrification by rhizobacteria diminishes the amount of NO3- available for plant nutrition, it may have positive effects on root development by means of NO production. Thus, NO produced by *A. brasilense* during root colonization promotes

the formation of lateral and adventitious roots (Creus *et al.* 2005; Molina-Favero *et al.* 2008).

b. Bacterial VOCs

Some PGPR, in particular *B. subtilis, B. amyloliquefaciens,* and *Enterobacter cloacae* promote plant growth by emitting VOC (Ryu *et al.* 2003). VOC are compounds of low molecular weight that are in a gaseous form under standard atmospheric and temperature conditions, and they include hydrocarbons, alcohols, aldehydes, and ketones. Among the huge diversity of VOC, 2,3-butanediol and acetoin have been the most studied and display a high growth-promoting effect (Kai *et al.* 2009). As bacteria emit a complex mixture of VOC and as genetic determinants involved in their biosynthesis are not all characterized yet, the biological activity of each compound is often difficult to evaluate. In order to understand how bacterial VOC stimulate plant growth promotion, the transcriptomic response of *Arabidopsis thaliana* to VOC emitted by *B. subtilis* GB03 was investigated (Zhang *et al.* 2007). This study reported the differential expression of genes related to cell wall modifications, primary and secondary metabolism, stress responses and auxin homeostasis, and suggested that VOC can directly affect pathways involved in plant morphogenesis. It is worth noting that PGPR's VOC are also involved in plant protection both by (i) triggering ISR pathways in the plant (Kloepper *et al.* 2004; Ryu *et al.* 2004), and by (ii) inhibiting the growth of plant pathogens (Haas *et al.* 2002).

c. Unusual effects of bacterial compounds on plants

Among molecular signals released in the rhizosphere, DAPG is an intriguing compound synthesized by *Pseudomonas* PGPR. DAPG-producing PGPR are well known for their capacity to suppress diverse soilborne diseases linked to their antifungal properties (Couillerot *et al.* 2009; Weller 2007). This phenolic compound was also found to be antibacterial, antiviral, antihelminthic, and phytotoxic when used at a high concentration (Haas and Keel 2003; Weller 2007). However, at lower concentrations, DAPG can also be a signal molecule for plants, inducing systemic resistance (Bakker *et al.* 2007; Iavicoli *et al.* 2003), stimulating root exudation (Phillips *et al.* 2004), and enhancing branching of the root system (Brazelton *et al.*

2008; Couillerot *et al.* 2011; Walker *et al.* 2011). Brazelton and collaborators (2008) demonstrated that applications of exogenous DAPG, at a concentration around 20 µM -which corresponds to that found in the rhizosphere of plants inoculated with DAPG⁺ *Pseudomonas* PGPR- inhibited primary root growth, stimulated lateral root production in tomato seedlings, and inhibited the activation of a reporter gene fusion under the control of an auxin-inducible promoter in transgenic tobacco hypocotyls. DAPG can indeed alter crop root architecture by interacting with an auxin-dependent signalling pathway. Furthermore, it was reported that the abundance of DAPG⁺ pseudomonads in agricultural soils was positively correlated with enhanced maize yields (Rotenberg *et al.* 2007).

Several studies clearly show that bacterial QS molecules could act as signals outside the bacterial kingdom; notably, bacterial AHLs trigger specific responses in plants. The first evidence of plant susceptibility to AHL has been observed with *Medicago truncatula* roots treated with nanomolar and micromolar concentrations of AHLs from both symbiotic (*Sinorhizobium meliloti*) and pathogenic (*Pseudomonas aeruginosa*) bacteria. *M. truncatula* responded by significant changes in the accumulation of over 150 proteins, including proteins involved in defense and stress response, flavonoid metabolism and hormone response and metabolism (Mathesius *et al.* 2003). In addition, exposure to AHLs induced changes in the secretion of plant compounds that mimic QS signals and thus have the potential to disrupt QS in associated bacteria (Mathesius *et al.* 2003).

Inoculation of tomato roots with AHL producers such as *Serratia liquefaciens* or *Pseudomonas putida* increased systemic resistance against the fungal leaf pathogen, Alternaria alternata; the *S. liquefaciens* AHL- mutant was less effective in reducing symptoms. Salicylic acid (SA) levels were increased in leaves when AHL-producing bacteria colonized the rhizosphere. Furthermore, AHL molecules systemically induced SA- and ethylene-dependent defense genes (Schuhegger *et al.* 2006). Induced systemic resistance to *Botrytis cinerea* in bean and tomato was also attributed to AHL signalling (Pang *et al.* 2009). These data support the view that AHL molecules, per se, play a role in the biocontrol activity of rhizobacteria through the induction of systemic resistance to pathogens.

The contact of *Arabidopsis thaliana* roots with 10 µM of C6-HSL resulted in distinct transcriptional changes in roots and shoots (von Rad *et al.* 2008). Several genes associated with cell growth as well as genes regulated by hormones showed changes in their expression level. The treatment of roots with different AHLs (ranging from 4 to 10 carbons) showed that C4-HSL and C6-HSL could trigger root elongation whereas C10-HSL decreased growth of root and leaf rosette. Moreover, C6-HSL, taken up by the plant and distributed systemically, did induce a shift of the auxin: cytokinin ratio both in leaf and root tissues toward higher auxin levels (von Rad *et al.* 2008). Another study aiming at evaluating *A. thaliana* growth responses to a variety of AHLs showed that C_{10}-HSL was the most active AHL; treatment with 48 µM of C_{10}-HSL caused a 80% reduction in root primary length and promoted lateral root development, root hair density and root hair growth (Ortiz-Castro *et al.* 2008). Although the effects of C_{10}-HSL on root architecture were similar to those produced by auxins, the response to this compound was found to be independent of auxin signalling (Ortiz-Castro *et al.* 2008). In addition, C_{10}-HSL can modulate senescence-related processes, most likely by interacting with jasmonic acid signalling (Morquecho-Contreras *et al.* 2010). A more recent study carried out with 3oxo-C14-HSL showed that this AHL has no effect on root growth of *A. thaliana* and could not be transported systemically; interestingly, perception of this AHL significantly increased the resistance towards hemibiotrophic bacteria and biotrophic fungi via the activation of mitogen-activated protein kinases, while it appeared rather ineffective against microbes with a necrotrophic life style (Schikora *et al.* 2011).

On mung bean, 3-oxo-C_{10}-HSL, in contrast to its analogue C_{10}-HSL, efficiently stimulated the formation of adventitious roots and the expression of auxin-response genes, possibly via $H_2O_2^-$ and NO^- dependent cGMP signalling (Bai *et al.* 2012).

Altogether, these studies indicate that plants have evolved means to perceive AHLs and that plant responses are highly dependent on the nature and concentration of AHL. AHLs commonly produced by rhizobacteria are crucial factors for plant growth and plant pathology, and play important roles in the beneficial or pathogenic outcomes of eukaryote–prokaryote interactions.

Finally, diketopiperazines (DKP) might constitute another class of transkingdom signals; indeed, DKP of a *Pseudomonas aeruginosa* strain were recently shown to be involved in plant growth promotion by this bacterium and to possess auxin-like activity (modulation of root architecture and activation of auxin-regulated genes) (Ortiz-Castro *et al.* 2011). Interestingly, DKP biosynthesis is regulated by the LasI/LasR/3oxo-C_{12}-HSL QS system.

I.1.4. PGPR signals with effects on other PGPR

PGPR exchange several types of cell-to-cell communication signals between each other and with other rhizosphere-inhabiting bacteria and fungi. The best known are obviously QS compounds by which bacteria couple gene expression to population density and trigger a coordinate response only when the QS signals reach a critical threshold. In this part, the role of QS compounds in the regulation of PGPR plant growth-promotion and biocontrol properties will be specifically discussed. However, other bacterial compounds may act as signals and regulate bacterial properties independently of the cell density.

I.1.4.1. Cell-density dependent regulation of plant growth-promotion and biocontrol properties between PGPR

As previously mentioned (see above), bacterial AHLs can affect root architecture. However, AHL signals were initially described for their role in regulating specific phenotypes at high cell density in a given bacterial population. Various reports indicate that biocontrol properties of PGPR are regulated by AHL-mediated QS; notably the production of antifungal compounds such as phenazines by various pseudomonads is controlled by QS (Chin-A-Woeng *et al.* 2001; De Mayer *et al.* 2011; Pierson *et al.* 1994), as well as pyrrolnitrin in some rhizospheric biocontrol *Serratia plymuthica* and *Burkholderia* (Liu *et al.* 2007; Schmidt *et al.* 2009).

As for bacterial traits involved in phytostimulation (such as production of phytohormones or phosphate solubilization), there is scarce evidence of QS regulation. Only a few members of the genus *Azospirillum* display AHL production

(Vial *et al.* 2006); for one isolate, an endophyte isolated from rice, AHL inactivation abolished pectinase activity, increased siderophore synthesis and reduced indoleacetic acid production (in stationary phase) but had no deleterious effect on the phytostimulatory properties (Boyer *et al.* 2008). A global proteomic approach revealed that QS is likely dedicated to regulating functions linked to rhizosphere competence and adaptation to plant roots (Boyer *et al.* 2008). Negative QS regulation of indoleacetic acid production was also reported for an endophytic *Serratia plymuthica* isolate (Liu *et al.* 2011).

Early events involved in the PGPR-plant interaction can also be regulated by QS and impact biocontrol and plant growth-promotion properties. Indeed motility, rhizosphere colonization and/or biofilm formation were shown to be regulated by QS in *Pseudomonas putida* (Dubern *et al.* 2006; Steidle *et al.* 2002), in *P. fluorescens* (Wei and Zhang, 2006), in *P. chlororaphis* (syn. aureofaciens) (Maddula *et al.* 2006 and 2008) and in *S. plymuthica* (Pang *et al.* 2009; Liu *et al.* 2011).

Finally, in a natural habitat such as the rhizosphere, an AHL-producing bacterial species can coexist with other(s) species producing identical AHLs or structurally related AHL, and can perceive these AHLs, leading to cross-talk. Interpopulation signalling was indeed evidenced in the rhizosphere of wheat and tomato (Pierson *et al.* 1998; Steidle *et al.* 2001 Wood *et al.* 1997) and can thus have a major impact on QS-regulated properties of PGPR.

I.1.4.2. Cell-density independent regulation of biocontrol properties and plant growth-promotion properties between PGPR

Several transcriptomic studies have recently revealed that antimicrobial compounds might act as signals in natural environments modulating gene expression in bacteria and facilitating intra- and interspecies interactions within microbial communities (Romero *et al.* 2011; Wecke and Mascher 2011). This is the case for antimicrobial compounds such as DAPG and pyoluteorin, which influence gene expression of biocontrol traits in biocontrol pseudomonads (Baehler *et al.* 2005; Brodhagen *et al.* 2004) and plant growth-promotion properties in *Azospirillum* PGPR (Combes-Meynet *et al.* 2011).

Certain biocontrol strains of *Pseudomonas* produce several antimicrobial metabolites with broad-spectrum antifungal activity such as *P. protegens* CHA0 which synthesizes DAPG, pyoluteorin (PLT) and pyrrolnitrin (PRN) (Haas and Keel, 2003). By using reporter fusions with the promoter regions of operons directing the biosynthesis of DAPG, or PLT, several reports show that DAPG and PLT induce, at a transcriptional level, their own biosynthesis while they repress the synthesis of the other metabolite for concentrations ranging from 10 to 100 µM (Baehler *et al.* 2005; Brodhagen *et al.* 2004; Maurhofer *et al.* 2004). PRN does not induce its own biosynthesis and is not involved in the regulation of the DAPG–PLT balance. Antifungal compounds produced by biocontrol *Pseudomonas* can thus act as signals mediating communication between *Pseudomonas* populations producing these antimicrobials.

Recently, we showed that DAPG can also act as a signal mediating inter-population communication between *Pseudomonas* and *Azospirillum* PGPR. By using a differential fluorescence induction promoter-trapping approach based on flow cytometry, we found that DAPG at concentrations of 0.1 and 10 µM induced a wide range of genes in *A. brasilense*, noticeably genes involved in phytostimulation such as *ppdC* (auxin production), *nirK* (NO production), and *nifX-nifB* (nitrogen fixation), and in root colonization (with genes involved in cell motility, biofilm formation, and poly-β-hydroxybutyrate production). On roots, these plant-beneficial genes are upregulated in the presence of the DAPG-producing *P. fluorescens* F113 strain compared with its DAPG-negative mutant (**Figure 4**), leading thereby to enhanced phytostimulatory effects of *A. brasilense* Sp245 on wheat when co-inoculated with *P. fluorescens* F113 (Combes-Meynet *et al.* 2011).

A wide diversity of plant- and bacterial-derived chemical signals occurs in the rhizosphere. As underlined in this review, they play a major role in the establishment of associative symbiosis between plants and PGPR. Some rhizosphere signals like phytohormones can be similarly produced by plants and PGPR. But, they may display distinct effects on rhizosphere inhabitants according to their concentrations, and to the organisms that will perceive them.

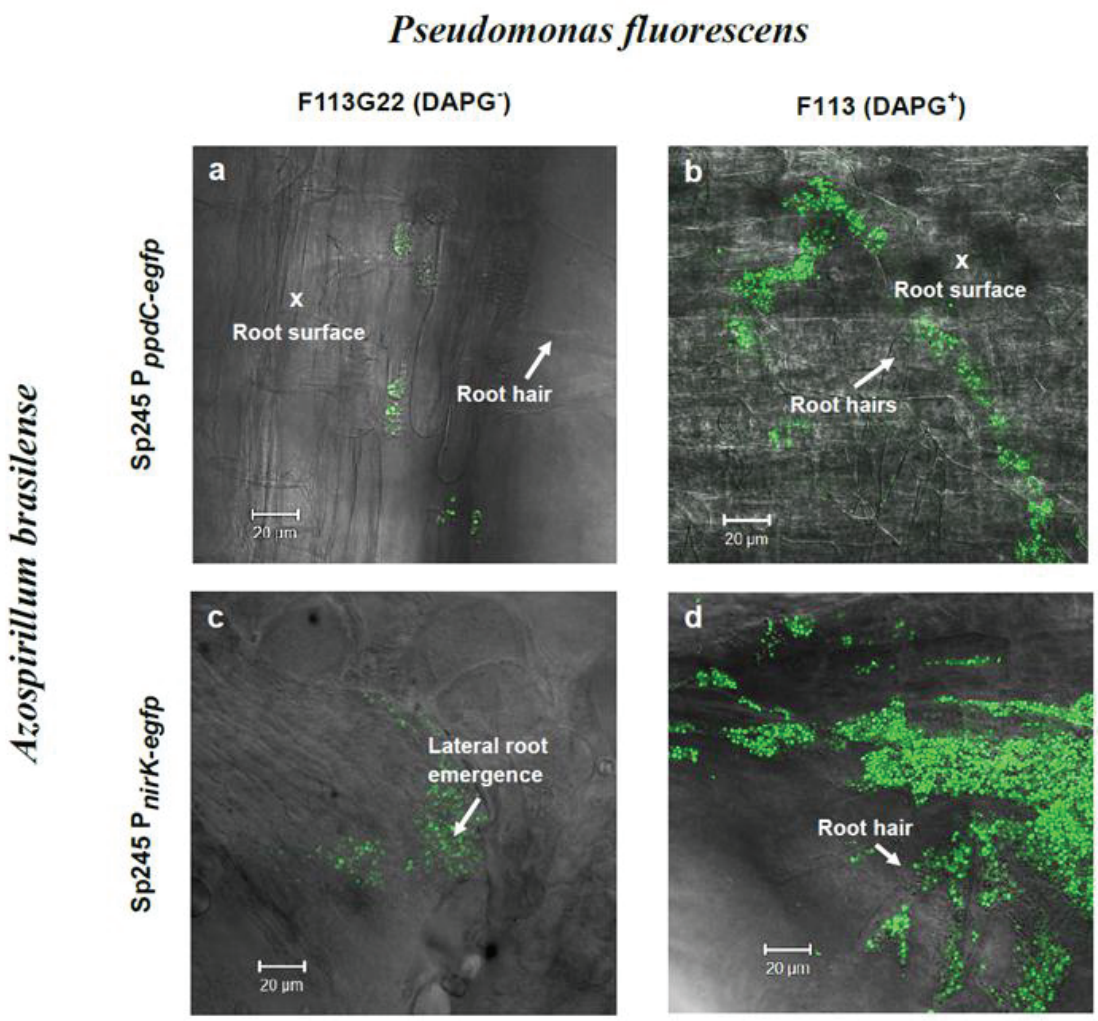

Figure 4 : Confocal laser scanning microscope images of *A. brasilense* Sp245-Rif DFI clones.

A. brasilense Sp245-Rif DFI clones expressing the *ppdC-egfp* (a, b) and the *nirK-egfp* (c, d) fusions on wheat roots at 7 d after co-inoculation with *P. fluorescens* F113 (b, d) and its DAPG-negative mutant F113G22 (a, c). Observations were made using a 510 Meta microscope (Carl Zeiss S.A.S., Oberkochen, Germany) equipped with an argon-krypton laser, detectors and filter sets for green fluorescence (i.e. 488 nm for excitation and 510-531 nm for detection). Cells expressing EGFP are green and grey backgrounds correspond to the root image formed by the transmitted light. The same detector amplification gains were used for semi-quantitative comparison of fluorescence levels in the three treatments for each DFI clone. Images are representative of the analysis of at least 10 images per condition. *A. brasilense* Sp245's *nirK* and *ppdC* genes are upregulated in the presence of the DAPG-producing *P. fluorescens* F113 strain (b, d), evidencing a positive signal effect of DAPG on the expression of *A. brasilense* plant-beneficial genes (see Combes-Meynet *et al.*, 2011).

I.1.5. Concluding remarks

The rhizosphere can be viewed as a mosaic of interconnected microhabitats where distinct concentration gradients of molecules are found. In this context, some rhizosphere molecules such as the antimicrobial compound DAPG can contribute, at high concentrations, to keep away potential bacterial and fungal competitors from *Pseudomonas* producers, and, at lower concentrations, i.e. further away from these *Pseudomonas* producers, to increase the rhizocompetence and the beneficial relationships of other microbial partners. It becomes obvious that taking into account the spatial distribution of PGPR populations in the rhizosphere could provide a better understanding of their behavior, their beneficial activities, and their relationships with other rhizosphere inhabitants (**Figure 5**).

We are far from understanding the whole diversity of rhizosphere signals, and forthcoming research will undoubtedly enable the scientific community to propose new natural products and strategies for growing crops in a sustainable way.

Acknowledgements

We thank EU project MicroMaize and ANR Blanc project AzoRiz (ANR-08-BLAN-0098) for funding. B.D. received a Ph.D. fellowship from "Région Rhône-Alpes".

Figure 5: Representation of major signaling events occurring between plant roots and PGPR or between PGPR themselves in the rhizosphere of crops.
Representation of major signaling events occurring between plant roots and PGPR or between PGPR themselves in the rhizosphere of crops. The rhizosphere contains a huge diversity of plant (indicated in green) or PGPR-derived (indicated in red or blue) compounds whose concentrations will vary in the rhizosphere habitat according particularly to the distance from their emission point. These compounds can act as signals, and may be involved in the control of root system architecture (RSA) and of phytostimulatory and biocontrol activities of PGPR. Positive effects are indicated by arrows whereas negative effects are indicated by lines terminated by a perpendicular bar. Some rhizosphere molecules such as DAPG might contribute, at high concentrations, to keep away potential bacterial and fungal competitors from biocontrol PGPR and, at lower concentrations, i.e. further away from producers, to stimulate the plant-beneficial properties of phytostimulatory PGPR. Taking into account the spatial distribution of PGPR populations in the rhizosphere is an important issue to better understand the functional activities and relationships of PGPR with other rhizosphere inhabitants. AHL: *N*-acyl-homoserine lactone, DAPG: 2,4-diacetylphloroglucinol, GA: gibberellic acid, IAA: indole-3-acetic acid, NO: nitric oxide, PLT: pyoluteorin, VOC: volatile organic compounds.dans http://news.harvard.edu/).

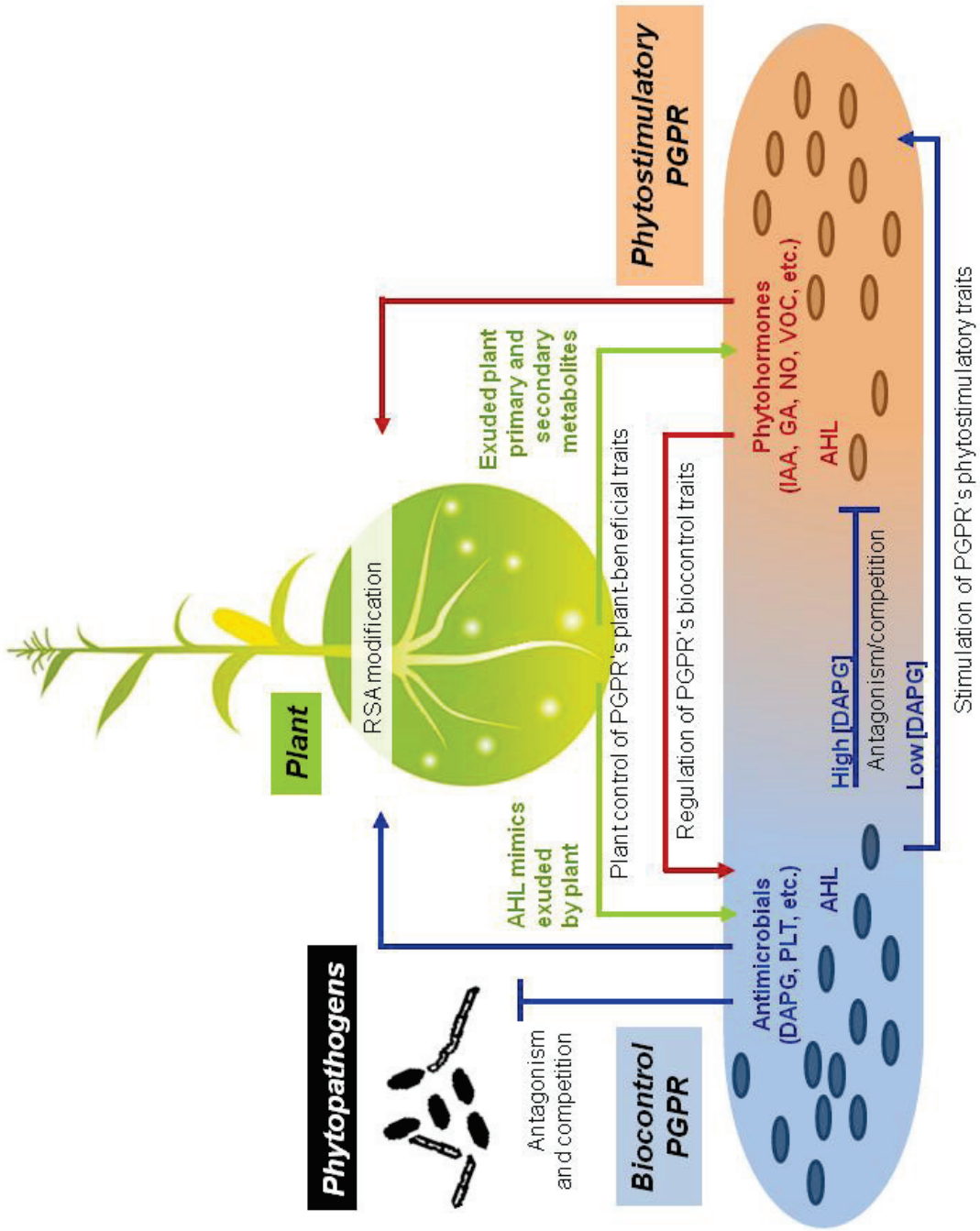

Figure 5 : Representation of major signaling events occurring between plant roots and PGPR or between PGPR themselves in the rhizosphere of crops.

Partie 2 :

Quelle spécificité dans la coopération entre les rhizobactéries phyto-stimulatrices et les plantes ?

Benoît Drogue, Hugo Doré, Stéphanie Borland, Florence Wisniewski-Dyé et Claire Prigent-Combaret

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Which specificity in the cooperation between phytostimulating rhizobacteria and plants?

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are found in association with a large range of host plants. If the question of plant host specificity has been well addressed in parasitic and mutualistic interactions, whether or not phytostimulating rhizobacteria efficiently interact only with specific host remains poorly discussed. This review presents elements suggesting the existence of specificity in the three-step establishment of the associative symbiosis between phytostimulating rhizobacteria and plants: bacterial attraction by the host plant, bacterial colonization of roots, and functioning of associative symbiosis.

Keywords: Adherence, cooperation, chemotaxis, plant growth-promoting rhizobacteria, plant genotype, host specificity

DOI : 10.1016/j.resmic.2012.08.006

Partie 3 :

Immunité des plantes et perception des PGPR

I.3.1. Introduction

Les plantes interagissent continuellement avec un nombre important de microorganismes dont la croissance est supportée par les composés organiques libérés par les racines (rhizodépots). De par leur composition, les rhizodépots modifient la chimie du microenvironnement proche des racines et participent à l'établissement d'une communauté rhizosphérique spécifique (Berg et Smalla 2009 ; Hartmann *et al.* 2009). Pour accéder à l'eau et aux nutriments contenus dans les végétaux, certains microorganismes vont jusqu'à envahir certains tissus de la plante et établir des interactions intimes et durables aux issues variées (Soto *et al.* 2009). Dans le cas du mutualisme, l'interaction conduit généralement à la formation d'un organe spécifique, siège d'un échange nutritionnel dont les coûts et les bénéfices sont partagés de manière réciproque par les deux partenaires (Odum 1971 ; Masson-Boivin *et al.* 2009 ; Smith et Read 2008). Dans le cas du parasitisme, l'interaction est défavorable au partenaire végétal et le microorganisme se développe aux dépends de la croissance et de la reproduction de la plante (O'Brien *et al.* 2011 ; Schumacher et Tundzynski 2012). Qu'il s'agisse du parasitisme ou du mutualisme, le partenaire microbien est perçu comme un intrus et le succès de l'interaction dépend de sa capacité à outrepasser les défenses de la plante (Soto *et al.* 2009).

Comme pour le mutualisme, les symbioses associatives impliquant les PGPR conduisent à une amélioration de la croissance et de la santé des plantes (Lugtenberg et Kamilova 2009 ; Vessey 2003). En revanche, aucun organe spécifique n'est formé par les partenaires de l'interaction et toutes les bactéries PGPR n'envahissent pas les tissus végétaux (Lugtenberg et Kamilova 2009 ; Monteiro *et al.* 2012). Si les mécanismes bactériens impliqués dans les effets phytobénéfiques ont été largement étudiés, la plupart des analyses évalue l'impact sur la morphologie de la plante et peu d'études se sont intéressées aux changements induits à l'échelle moléculaire. Ainsi, les mécanismes végétaux impliqués dans la perception des PGPR et le rôle des systèmes de défense dans l'établissement d'une coopération durable et efficace sont rarement traités.

Dans ce contexte, l'objectif de cette partie est de synthétiser les connaissances actuelles sur l'impact des PGPR sur le partenaire végétal, du point de vue des mécanismes moléculaires mis en oeuvre. Après une brève description des mécanismes impliqués dans la perception des bactéries pathogènes et mutualistes, nous nous intéresserons aux études portant sur l'influence des PGPR sur l'immunité et les signaux végétaux.

I.3.2. Immunité des plantes et perception des bactéries pathogènes et mutualistes

Afin de limiter leur invasion par des organismes étrangers, les plantes ont développé des barrières structurelles et métaboliques (paroi cellulaire, molécules antimicrobiennes…) qui constituent les premiers remparts de protection face aux pathogènes (Pieterse *et al.* 2009). Derrière cette première ligne de défense, le système immunitaire de la plante permet de limiter l'invasion par les microorganismes qui tentent de pénétrer les tissus (Jones et Dangl 2006).

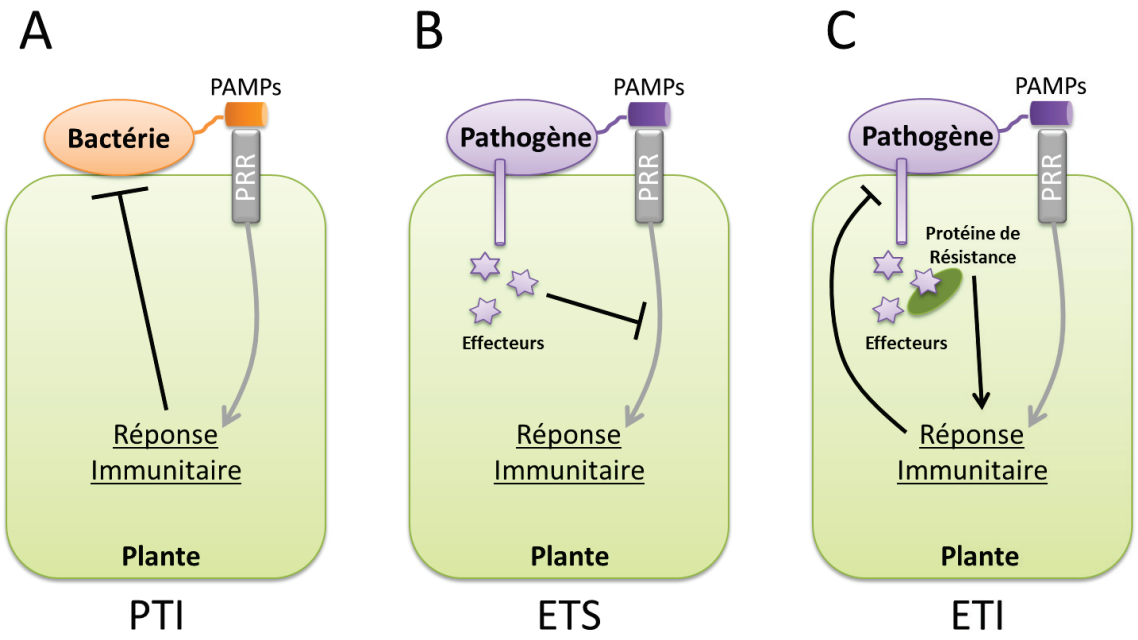

Figure 6 : Système immunitaire des plantes et perception des pathogènes.
A, l'immunité déclenchée par les MAMPs/PAMPs (PTI) permet de limiter la colonisation des tissus végétaux par les microorganismes. B, les pathogènes produisent des effecteurs capables d'interférer avec la PTI, conduisant à la sensibilité de l'hôte végétal (ETS). C, les plantes sont capables de reconnaitre spécifiquement certains effecteurs par l'intermédiaire des protéines de résistance qui induisent une réponse immunitaire secondaire (ETI).
Adapté d'après Pieterse *et al.* 2009.

Dans un premier temps, ce système fait intervenir la reconnaissance de déterminants microbiens universels tels que la flagelline, la chitine, les glycoprotéines, le peptidoglycane ou encore les lipopolysaccharides, regroupés sous le terme de Motifs Moléculaires Associés aux Microorganismes (PAMPs pour *Pathogens–Associated Molecular Patterns*) (Schwessinger et Zipfel 2008). Les PAMPs activent des Récepteurs de Reconnaissance des Motifs Moléculaires (PRRs pour *Pattern Recognition Receptors*) qui initient la réponse immunitaire. Cette immunité déclenchée par les PAMPs (PTI pour PAMPs-*Triggered Immunity*) permet de limiter la colonisation des tissus végétaux par les microorganismes (Chisholm *et al.* 2006 ; Nurnberger *et al.* 2004) (**Figure 6 A**). Elle se traduit par l'induction des gènes PR (PR pour *Pathogenesis-Related*), la production d'espèces réactives de l'oxygène (ROS), de métabolites secondaires antimicrobiens (phytoalexines) et le renforcement de la paroi cellulaire (Chisholm *et al.* 2006 ; Pieterse *et al.* 2009).

Dans le cas du parasitisme, les bactéries se soustraient au système immunitaire de la plante par l'intermédiaire d'effecteurs sécrétés directement dans les cellules hôtes (**Figure 6 B**). Ces effecteurs interfèrent avec les voies de signalisation intracellulaire et conduisent à l'évitement de la PTI (Chisholm *et al.* 2006 ; Pieterse *et al.* 2009). Ce mécanisme de sensibilité déclenché par les effecteurs (ETS pour *Effector-Triggered Susceptibility*) permet l'établissement du pathogène dans la plante hôte (Chisholm *et al.* 2006 ; Jones et Dangl 2006). Par l'intermédiaire des protéines de résistance, les végétaux sont capables de reconnaitre spécifiquement les effecteurs de certains pathogènes (Chisholm *et al.* 2006 ; Jones et Dangl 2006). Ce mécanisme conduit à une réponse immunitaire secondaire déclenchée par les effecteurs (ETI pour *Effector-Triggered Immunity*) (**Figure 6 C**). L'issue de la « bataille » dépend alors de la balance entre la capacité du pathogène à éviter les défenses de la plante et la capacité de la plante à reconnaître l'intrus et activer des défenses efficaces (Pieterse *et al.* 2009). On parlera d'interaction compatible lorsque le pathogène est capable d'induire la maladie et d'interaction incompatible lorsque la plante se défend efficacement. Si les voies de signalisation impliquées dans les réponses de types PTI et ETI sont similaires, la réponse de type ETI est plus drastique et conduit à une mort cellulaire programmée, localisée au site d'infection (Pieterse *et al.* 2009 ; Soto *et al.* 2009 ; Tsuda *et al.* 2008). Cette réponse

hypersensible permet d'isoler le pathogène du reste de la plante et de prévenir les dommages supplémentaires (De Wit 1997). Notons que la distinction dichotomique entre PTI et ETI est aujourd'hui controversée car de nombreux exemples montrent que l'induction de la réponse hypersensible n'est pas restreinte à la réponse ETI, suggérant l'existence d'un continuum entre PTI et ETI (Thomma *et al.* 2012).

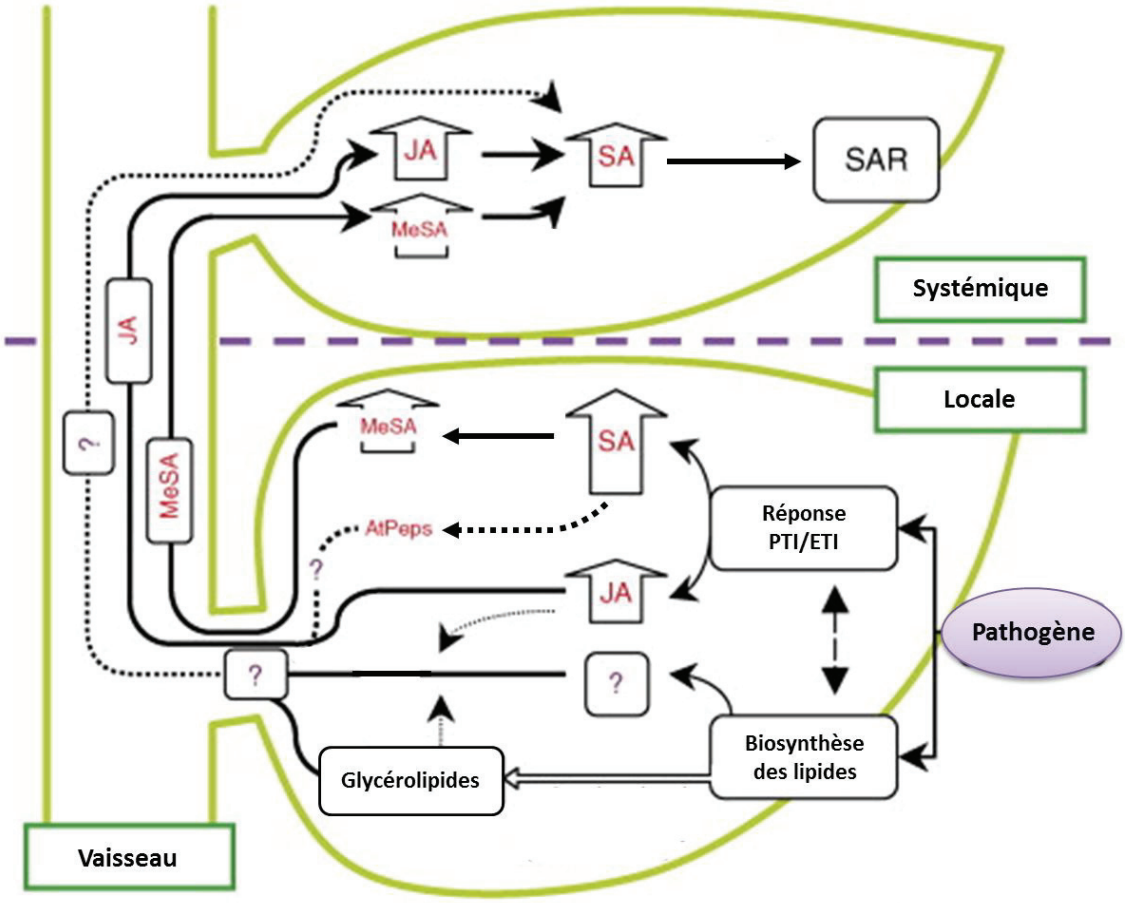

Figure 7 : Transport du signal au cours de la Réponse Systémique Acquise (SAR).
La perception du pathogène induit des réponses immunitaires locales de type PTI ou ETI. L'accumulation d'acide salicylique (SA) induit une réponse systémique : la SAR. Le jasmonate (JA) et le méthyle salicylate (MeSA) constituent les signaux mobiles impliqués dans la réponse systémique. Un facteur dérivé des glycérolipides et un groupe de peptides (AtPeps) pourraient également constituer des signaux mobiles.
Simplifié d'après Vlot *et al.* 2008.

Les phytohormones jouent un rôle important dans les réseaux de signalisation cellulaire impliqués dans l'établissement d'une réponse immunitaire efficace (Pieterse *et al.* 2009). Au-delà des réponses locales, elles peuvent induire des réponses systémiques permettant de protéger les tissus végétaux sains, distants du site d'infection. Ainsi l'acide salicylique contribue à l'établissement de la Résistance Systémique Acquise (SAR) (Grant et Lamb 2006) (**Figure 7**). La SAR correspond à une résistance durable exercée contre un large spectre de pathogènes qui conduit à l'expression systémique des gènes PR. Ces gènes codent pour des protéines, dites protéines PR (*Pathogenesis-Related*), qui présentent des activités antimicrobiennes de type chitinase, peroxydase, glucanase ou encore défensine (Sels *et al.* 2008). Plusieurs études montrent que si la mise en place de la SAR nécessite l'accumulation d'acide salicylique au site d'infection et dans les tissus distants, ce composé n'est pas le signal mobile impliqué dans la réponse systémique (Vernooij *et al.* 1994 ; Vlot *et al.* 2008). Le transport du signal à travers la plante ferait notamment intervenir le méthyle salicylate (un dérivé de l'acide salicylique), le jasmonate, ainsi qu'un facteur dérivé des glycérolipides et un groupe de peptides impliqué dans la signalisation de cellule à cellule (Vlot *et al.* 2008) (**Figure 7**).

Dans le cas du mutualisme, la perception précoce des rhizobia présente des similitudes avec les réponses immunitaires de type PTI et ETI induites par les pathogènes (Soto *et al.* 2009). Cependant, cette réponse est plus mesurée que dans le cas du parasitisme et ne conduit pas à la réponse hypersensible. Ceci s'expliquerait en partie par le rôle des facteurs Nod dans le contrôle de l'accumulation locale d'acide salicylique (Martínez-Abarca *et al.* 1998 ; Soto *et al.* 2009). En effet, aucune accumulation significative d'acide salicylique n'a été rapportée dans le cas des interactions entre les légumineuses et des rhizobia compatibles (Soto *et al.* 2009). En revanche, une augmentation de la quantité d'acide salicylique a été détectée dans les racines de luzerne, suite à l'inoculation de rhizobia incapables de produire des facteurs Nod (Martínez-Abarca *et al.* 1998). D'autres résultats tendent à montrer que les signaux de défense impliquant l'acide salicylique jouent un rôle dans le contrôle de la nodulation. Ainsi, alors que l'application exogène d'acide salicylique retarde et inhibe la formation des nodosités, la réduction de sa concentration endogène chez des plantes transgéniques conduit à une

augmentation de la nodulation (van Spronsen *et al.* 2003 ; Stacey *et al.* 2006).

D'autres similitudes ont été observées entre les étapes précoces du parasitisme et celles du mutualisme (Soto *et al.* 2009). C'est par exemple le cas de la production de ROS par la plante dont le rôle dans la progression de l'interaction mutualiste reste à clarifier. Du point de vue de la bactérie, les effecteurs protéiques associés aux systèmes de sécrétion de type III et IV peuvent jouer un rôle important dans la modulation des défenses de la plante.

I.3.3. Immunité des plantes et perception des PGPR

La santé des plantes est fortement influencée par l'établissement de bactéries coopératrices dans la rhizosphère. Alors que certaines PGPR phytoprotectrices sont perçues comme des éliciteurs de défense, les mécanismes impliqués dans la perception des PGPR phytostimulatrices par les plantes restent peu étudiés.

Ainsi, la perception des PGPR phytoprotectrices peut conduire à l'induction d'une résistance systémique durable et efficace contre un large spectre de pathogènes, appelée ISR (pour Induced Systemic Resistance) (Pieterse *et al.* 2009 ; Van Wees *et al.* 2008 ; Van Loon *et al.* 1998) (**Figure 8**). Contrairement à la SAR qui dépend de voies de signalisation impliquant l'acide salicylique, l'ISR fait intervenir des voies associées au jasmonate et à l'ethylène. Alors que la SAR conduit à une induction directe des systèmes de défense, l'ISR correspond à un amorçage de la réponse immunitaire qui permet à la plante de répondre plus efficacement vis-à-vis de l'infection ultérieure par des pathogènes (Pieterse *et al.* 2009). Ainsi, l'effet d'amorçage par les PGPR n'est généralement pas associé à une reprogrammation conséquente du transcriptome végétal et les changements systémiques sont relativement faibles (Van Wees *et al.* 2008 ; Verhagen *et al.* 2004 ; Wang *et al.* 2005). En revanche, l'amorçage permet une mise en place plus rapide et plus intense des défenses de la plante en réponse à l'infection par un pathogène (Pieterse *et al.* 2009 ; Verhagen *et al.* 2004). L'ISR se traduit alors par l'induction efficace des gènes contrôlés par le jasmonate et l'éthylène, notamment des gènes codant des

défensines, ainsi que par la mise en place d'un dépôt de callose et de lignine au site d'infection (Van Wees *et al.* 2008 ; Verhagen *et al.* 2004).

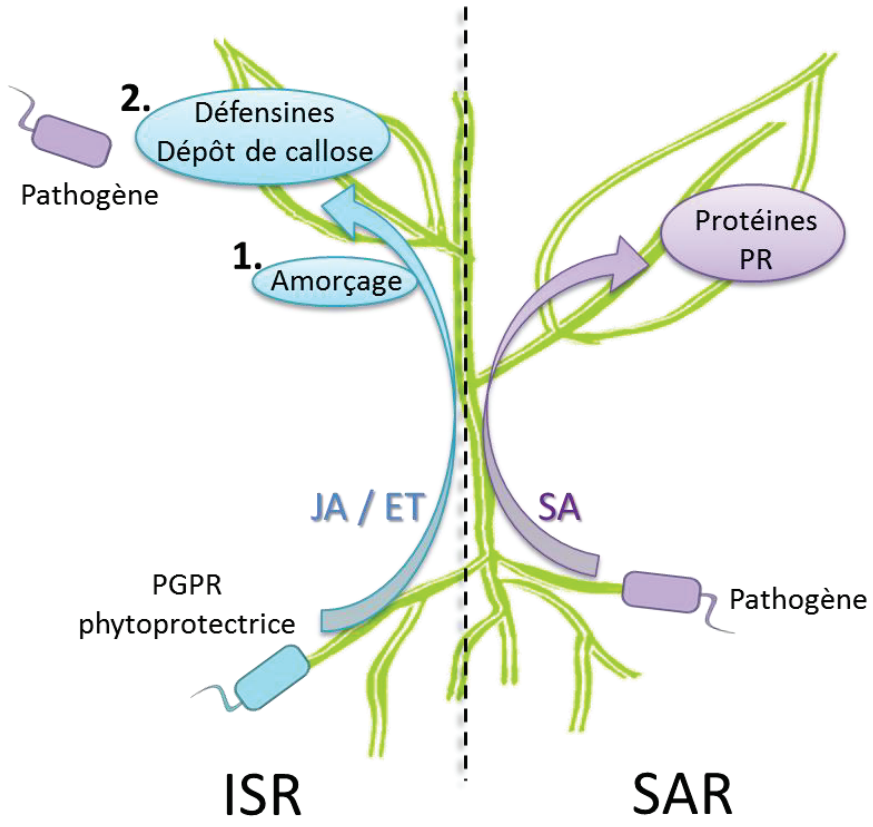

Figure 8 : Représentation schématique des réponses immunitaires systémiques.
La perception des PGPR phytoprotectrices peut induire une réponse systémique de type ISR (Induced Systemic Resistance). Contrairement à la SAR induite qui implique l'acide salicylique (SA) et les protéines PR, l'ISR implique le jasmonate (JA) et l'éthylène (ET) dans une réponse en deux phases : un effet d'amorçage (1.) qui conduit à une réponse rapide et efficace (2.) contre l'infection ultérieure par les pathogènes.

Dans le cas des PGPR phytostimulatrices, les systèmes de défenses des plantes sont principalement étudiés pour leur impact sur l'établissement de la coopération. Ainsi, une étude menée sur des bactéries du genre *Azospirillum* montre que leur inoculation n'entraine pas de réaction d'hypersensiblité et que les niveaux de phytoalexine produits par les plantes inoculées restent bas, contrairement à ceux induits par des pathogènes compatibles (Bashan 1998). Chez la vigne, la bactérie endophyte *Burkholderia phytofirmans* PsJN induit l'expression des gènes de défense

régulés par le jasmonate et l'acide salicylique, mais les niveaux d'induction sont plus faibles que ceux observés en réponse au pathogène *Pseudomonas syringae* pv. *pisi* (Bordiec *et al.* 2011). Ainsi les PGPR phytostimulatrices ne semblent pas perçues par les plantes comme des organismes pathogènes. Dans le cas des endophytes, l'induction des systèmes de défense semble dépendre du génotype de plante et contrôler l'établissement des interactions compatibles et incompatibles (Miché *et al.* 2006 ; Rosenblueth et Martínez-Romero 2006 ; Reinhold-Hurek et Hurek 2011). En effet, au cours de l'interaction entre *Azoarcus* et le riz, la colonisation d'un cultivar incompatible conduit à une surexpression plus importante des protéines de défense induites par le jasmonate (Miché *et al.* 2006). Dans le cas de l'association entre *Herbaspirillum* et les racines de riz, la répression des thionines et d'une protéine induite par le probénazole (PBZ1) suggère que la bactérie module les défenses de la plante à son avantage (Brusamarello-Santos *et al.* 2007). L'interaction entraine également la régulation des gènes de réponse à l'auxine et des gènes de réponse à l'éthylène.

Il est important de noter que la frontière entre PGPR phytostimulatrices et PGPR phytoprotectrices ainsi que leurs impacts respectifs sur le système immunitaire des plantes semblent plus modérés que ce qui est habituellement décrit. En effet, des résultats obtenus sur le riz montrent que l'inoculation de la bactérie phytostimulatrice *Azospirillum sp.* B510 induit une résistance contre le feu bactérien et la pyriculariose (Yasuda *et al.* 2009). Cette résistance n'est pas liée à l'induction des gènes PR ni des voies de signalisation impliquant l'acide salicylique ce qui suggère une action par un effet d'amorçage similaire à celui observé dans le cas d'une ISR. D'autre part, l'induction modérée des gènes associés au jasmonate et à l'acide salicylique par la bactérie *B. phytofirmans* PsJN confère également à la vigne une résistance contre le champignon pathogène *Botrytis* (Bordiec *et al.* 2011). Ces deux exemples illustrent la complexité des coopérations phytobénéfiques entre les PGPR et les plantes ainsi que la diversité des signaux impliqués dans ces interactions. En particulier, les phytohormones jouent un rôle central à la fois dans la croissance et dans la réponse immunitaire des végétaux, à travers des voies de signalisation interconnectées (Pieterse *et al.* 2009) (**Figure 9**).

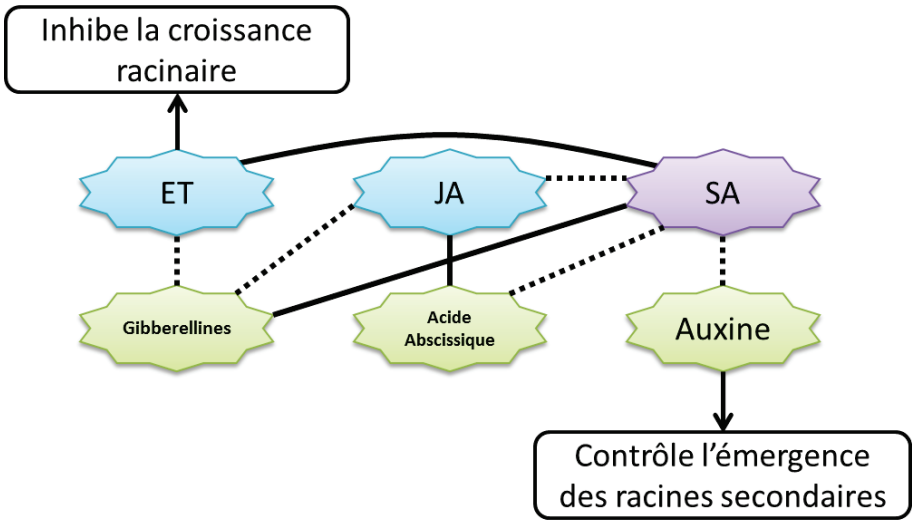

Figure 9 : Interconnexions entre les voies de signalisation impliquant les phytohormones.
Les formes de couleur symbolisent les voies de signalisation associées aux phytohormones impliquées principalement dans la SAR (violet), l'ISR (bleu), la croissance des plantes (vert). Les traits pleins symbolisent les interactions positives et les traits pointillés les interactions négatives entre les différentes voies de signalisation. Les flèches et les cadres noirs symbolisent l'effet des phytohormones sur la croissance végétale. ET, éthylène. JA, jasmonate. SA, acide salicylique.

A l'échelle systémique, les PGPR peuvent induire des modifications significatives de la composition en métabolites secondaires dont certains jouent un rôle essentiel dans les interactions plante-bactéries, parfois en lien avec les mécanismes de défense. Ainsi, plusieurs études ont mis en évidence l'impact de l'inoculation d'*Azospirillum* ou *Pseudomonas* sur la composition en métabolites secondaires des feuilles et des racines de maïs et de riz (Chamam *et al.* sous presse ; Walker *et al.* 2010, 2012). Sur le maïs, ces changements concernent principalement les benzoxazinoïdes dont certains présentent des propriétés antimicrobiennes contre *Agrobacterium tumefaciens* et *Erwinia* spp. (Corcuera *et al.* 1978 ; Sahi *et al.* 1990 ; Walker *et al.* 2010, 2012). Concernant le riz, les principales modifications observées touchent les flavonoïdes ou encore les acides hydroxycinnamiques dont certains jouent un rôle dans la résistance contre des insectes et des pathogènes fongiques (Chamam *et al.* sous presse, Leiss *et al.* 2009).

I.3.4. Conclusion

La perception des microorganismes par les plantes conduit à des réponses de défense de type PTI, ETS et ETI qui semblent former un continuum. Qu'il s'agisse du parasitisme, du mutualisme ou de la coopération, l'établissement durable des bactéries au contact de la plante nécessite une modulation permissive du système immunitaire ou son évitement. La transmission systémique du signal perçu lors de l'interaction avec des microorganismes fait intervenir des voies hormonales interconnectées impliquées à la fois dans la régulation des défenses et dans la croissance végétale. Dans la mesure où la modification de la balance hormonale des plantes peut contribuer de manière importante à l'effet bénéfique des PGPR phytostimulatrices (Bashan et de Bashan 2010), le rôle central des phytohormones et les interconnexions entre les différentes voies de signalisation sont autant de paramètres qu'il faut prendre en compte dans la compréhension des interactions PGPR-plantes.

Chapitre II :

Expression des gènes de la bactérie phytostimulatrice *Azospirillum lipoferum* 4B au cours de la coopération avec les céréales

Contexte

Les bactéries du genre *Azospirillum* sont les plus étudiées des PGPR phytostimulatrices et les mécanismes impliqués dans leurs effets phytobénéfiques sont bien décrits (**Figure 10**) (Bashan et de-Bashan 2010 ; Somers *et al.* 2004). Les principaux processus entrainant une amélioration de la croissance des plantes sont : la modification de la balance phytohormonale (auxines, gibberellines, cytokines, acide abscissique, éthylène, oxyde nitrique, désamination du 1-aminocyclopropane-1-carboxylate ou ACC), la fixation d'azote atmosphérique, l'augmentation de l'absorption hydrominérale et la solubilisation du phosphate, ainsi que l'atténuation des stress environnementaux (biotiques, hydriques, salins, pH, métaux toxiques…) (Bashan et de-Bashan 2010 ; Somers *et al.* 2004).

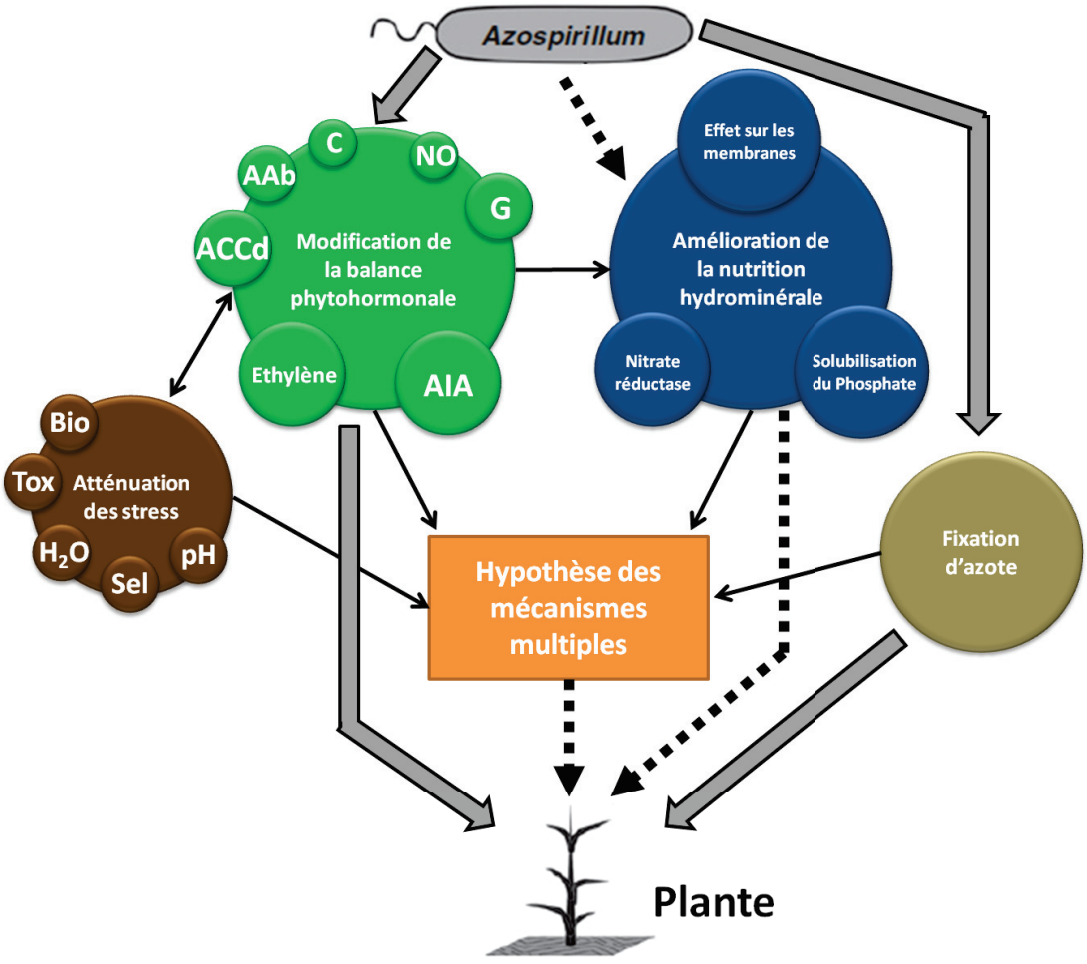

Figure 10 : Mécanismes impliqués dans l'effet phytobénéfique d'*Azospirillum*.
La taille des cercles représente l'importance relative de chaque mécanisme. Flèches grises : complexes enzymatiques ou molécules directement produites par la bactérie et dont l'impact sur la croissance des plantes a été démontré. Flèches noires pleines : Interactions entre les mécanismes. Flèches noires pointillées : hypothèses non démontrées. AAb, acide abscissique. ACCd, désamination de l'acide 1-aminocyclopropane-1-carboxylique. AIA, acide indole-3-acétique. C, cytokines. G, Gibbérellines. NO, oxyde nitrique. Tox, composés toxiques.
Adapté d'après Bashan et de-Bashan 2010.

Au cours des 40 dernières années, les effets positifs d'*Azospirillum* ont été principalement étudiés sur le riz, le maïs et le blé, céréales indispensables à l'alimentation animale et humaine (Bashan *et al.* 2004 ; Veresoglou et Menexes 2010). Les bénéfices économiques et environnementaux des bio-fertilisants ont mené à un accroissement des essais au champ, notamment en Inde et en Amérique Latine où plusieurs inoculum à base d'*Azospirillum* sont commercialisés (Bashan *et al.* 2004 ; Fuentes-Ramirez et Caballero-Mellado 2005). De nombreuses études rapportent que l'augmentation des rendements est proche de 10 % en moyenne, mais la variabilité entre les expériences est trop importante et le pourcentage de réussite insuffisant (60-70%) pour permettre la démocratisation de ces pratiques agricoles (Fuentes-Ramirez et Caballero-Mellado 2005 ; Okon et Itzigsohn 1995 ; Veresoglou et Menexes 2010). Cependant, peu d'études tiennent compte du fait qu'une souche particulière n'interagit pas de manière efficace avec toutes les plantes, dans tous les sols et quelles que soient les pratiques (Fuentes-Ramirez et Caballero-Mellado 2005). En particulier, l'importance des combinaisons entre génotypes bactériens et végétaux, suggérant l'existence d'une spécificité d'hôte, sont rarement considérées dans l'étude de la symbiose associative *Azospirillum*-céréales.

Tableau III : Caractéristiques génomiques d'*Azospirillum*[#].

Souches	Chromosome	p1	p2	p3	p4	p5	p6	TOTAL
***A. brasilense* CBG497**								
taille du réplicon	3 371 241	1 598 241	731 389	488 405	606 415[¶]	Absent	148 687	6 473 208
pourcentage G+C	68,0	68,7	68,8	66,05	69,3		67,1	68,4
nombre d'ORFs	3403	1430	643	12	583		122	6185
ARNr[¶]	nc	1	1	nc	nc		0	nc
ARNt	44	16	1	0	6		0	67
***A. brasilense* Sp245**								
taille du réplicon	3 023 440	1 766 028	912 449	778 798	690 334	191 828	167 364	7 530 241
pourcentage G+C	68,6	68,6	68,3	68,2	69,0	66,7	66,8	68,5
nombre d'ORFs	3309	1812	922	824	691	163	125	7846
ARNr	2	3	2	0	1	0	0	8
ARNt	44	25	2	0	9	0	1	81
***A. lipoferum* 4B**								
taille du réplicon	2 988 332	1 040 425	750 123	648 491	645 253	478 032	295 744	6 846 400
pourcentage G+C	67,6	67,6	67,6	67,8	68,3	67,7	67,1	67,7
nombre d'ORFs	2904	883	640	555	599	415	237	6233
ARNr	2	3*	2	1	0	1[†]	0	9
ARNt	46	12	5	2	6	8	0	79
***Azospirillum sp.* B510**								
taille du réplicon	3 311 395	1 455 109	723 779	681 723	628 837	537 299	261 596	7 599 738
pourcentage G+C	67,8	67,6	67,5	67,4	68,0	67,5	65,9	67,6
nombre d'ORFs	3287	1263	693	589	598	464	232	7126
ARNr	2	4	1	1	0	1	0	9
ARNt	45	14	2	3	6	9	0	79

[#] Adapté d'après Wisniewski-Dyé *et al.* 2012 (**Annexe II**).
*L'ARN 5S est absent d'un des opérons.
[†] L'ARN 23S est absent.
[¶] nc, non connu.

L'obtention récente de quatre génomes complets d'*Azospirillum* appartenant à trois espèces différentes (*Azospirillum* sp. B510, *A. lipoferum* 4B, *A. brasilense* Sp245, *A. brasilense* CBG497) a permis de définir le génome cœur d'*Azospirillum* (gènes partagés par toutes les souches), ainsi que les gènes spécifiques à chaque souche (**Tableau III**) (Kaneko *et al.* 2010 ; Wisniewski-Dyé *et al.* 2011, 2012 **AnnexeII**). L'ensemble des données génomiques disponibles a permis d'envisager des analyses transcriptomiques par puces à ADN afin d'identifier, d'une part, les gènes impliqués dans l'adaptation de la bactérie à sa plante hôte et, d'autre part, des déterminants génétiques potentiellement impliqués dans la spécificité d'hôte.

Notre choix s'est porté sur la souche *A. lipoferum* 4B, isolée du riz (cv. Cigalon) en Camargue (Thomas-Bauzon *et al.* 1982). Parmi ses principales caractéristiques, cette souche est capable de générer des variants à une fréquence élevée *in vitro* (10^{-4} à 10^{-3} par cellule et par génération) (Alexandre et Bally 1999). Cette variation phénotypique entraîne la perte d'un plasmide de 750kb (plasmide p2) qui porte notamment le gène *acdS*, codant une ACC désaminase (Vial *et al.* 2006 ; Prigent-Combaret *et al.* 2008). L'ACC est le précurseur de l'éthylène, une phytohormone qui inhibe la croissance des racines. Comme d'autres PGPR, *Azospirillum* est donc capable de dégrader l'ACC produit par la plante, et de diminuer la quantité d'ACC disponible pour la synthèse d'éthylène, entrainant une levée de l'inhibition racinaire exercée par cette phytohormone (Glick *et al.* 1998 ; Holguin et Glick 2003 ; Prigent-Combaret *et al.* 2008). De plus, *A. lipoferum* 4B héberge un phage inductible, comme c'est le cas de plusieurs autres souches d'*Azospirillum* (Boyer *et al.* 2008). Cette propriété pourrait expliquer l'incroyable quantité de transferts horizontaux de gènes observée dans les génomes d'*Azospirillum* (Wisniewski-Dyé *et al.* 2011). Enfin, de récentes études montrent que l'effet d'*Azospirillum* sur les céréales dépend de la combinaison souche/cultivar (Walker *et al.* 2011, Chamam *et al.* sous presse). Notamment, l'effet phytostimulateur de la souche *A. lipoferum* 4B est plus important sur son cultivar d'origine (cv. Cigalon) que sur un autre cultivar (cv. Nipponbarre) (Chamam *et al.* sous presse) (**Tableau IV**). De plus, l'inoculation d'*A. lipoferum* 4B sur les racines de riz entraine une modification cultivar-dépendante du métabolisme secondaire à la fois des racines et des feuilles (Chamam *et al.* sous presse).

Tableau IV : Effets d'*Azospirillum* sur le poids sec des racines et des tiges de riz*.

Traitement§	Poids sec des tiges (mg) #	Poids sec des racines (mg) #
Cv. Cigalon		
non inoculé	1,36 ± 0,08 b	1,31 ± 0,07 b
A. lipoferum 4B	1,97 ± 0,14 a	1,76 ± 0,14 a
Azospirillum sp. B510	1,14 ± 0,11 b	1,02 ± 0,07 b
Cv. Nipponbare		
Non inoculé	4,20 ± 0,77 b	2,12 ± 0,38 b
A. lipoferum 4B	5,23 ± 0,21 a	2,66 ± 0,11 a
Azospirillum sp. B510	5,43 ± 0,31 a	2,80 ± 0,19 a

§ A l'origine, la souche *A. lipoferum* 4B a été isolée du cv. Cigalon et la souche *Azospirillum sp.* B510 du cv. Nipponbare.

Les valeurs correspondent à la moyenne ± erreur standard, n=4. Pour chaque valeur et chaque cultivar, les differences statistiques entre les traitements sont indiqués par des lettres minuscules (analyse de variance et test de Fisher *least significant difference* ; P<0,05).

* Adapté d'après Chamam *et al.* sous presse.

Deux analyses distinctes ont été réalisées afin d'évaluer l'impact de la variabilité génotypique intraspécifique (**Partie 1**) et interspécifique (**Partie 2**) de la plante sur le transcriptome bactérien. Afin de s'affranchir de facteurs abiotiques et biotiques indépendants des deux partenaires de l'interaction, les inoculations ont été réalisées en système gnotobiotique sur des graines stériles. Ces systèmes ont été incubés dans des chambres climatiques afin de maitriser la photopériode, la température et l'humidité. La principale contrainte étant l'obtention d'une quantité suffisante d'ARN pour réaliser les analyses transcriptomiques, les bactéries au contact de la racine ont été récupérées sept jours après inoculation. En effet, à ce stade la taille des racines était optimale pour obtenir un nombre de bactéries, et par la suite une quantité d'ARN, suffisants. Les mises au point et les expériences d'inoculation ont été réalisées en collaboration avec Hervé Sanguin (Chercheur Post-Doctoral financé de juin 2009 à mai 2011 par le contrat ANR AZORIZ).

Dans la **Partie 1** nous avons cherché à caractériser les déterminants génétiques d'*A. lipoferum* 4B impliqués dans l'adaptation au riz. Afin de mettre en évidence une réponse hôte-spécifique à l'échelle du cultivar de riz (intraspécifique), la bactérie a été inoculée sur son cultivar d'origine (cv. Cigalon) et un cultivar génétiquement proche (cv. Nipponbare) (**Figure 11**). En parallèle, un témoin mimant une racine artificielle a été inoculé à partir de la même culture bactérienne. Ce témoin a permis de distinguer les gènes impliqués dans la transition de la vie planctonique (culture) à la vie sessile (bactéries adhérentes) de ceux plus particulièrement liés à l'adaptation à la plante hôte.

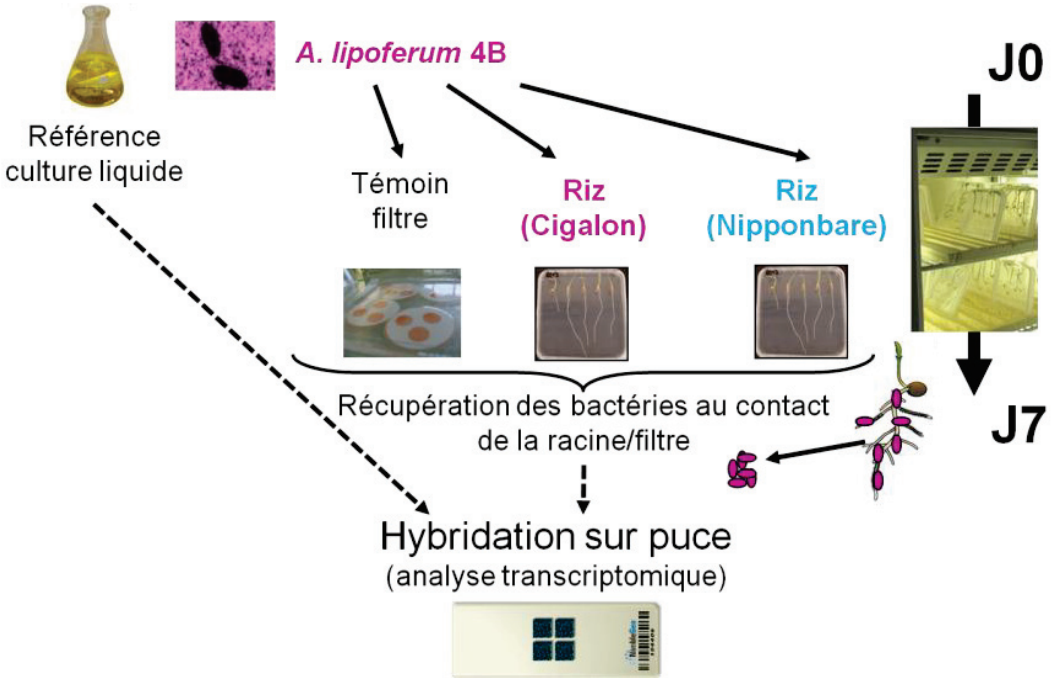

Figure 11 : Plan expérimental Chapitre II, Partie 1.

Dans la **Partie 2** nous nous sommes intéressés à la réponse hôte-spécifique à une échelle plus large, celle de la famille des *Poaceae* (Interspécifique). Pour cela, *A. lipoferum* 4B a été inoculée sur le riz, le maïs et le blé (**Figure 12**). Cette analyse nous a permis de mettre en évidence des gènes potentiellement impliqués dans la spécificité d'hôte de la coopération *A. lipoferum* 4B-céréales.

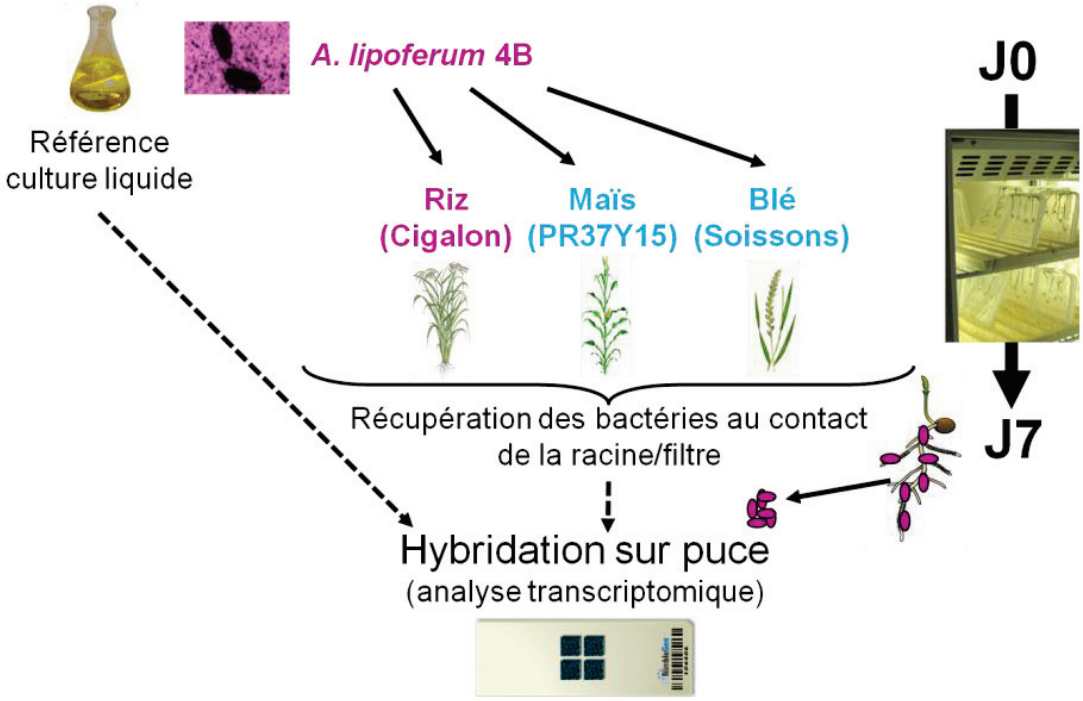

Figure 12 : Plan expérimental Chapitre II, Partie 2.

Partie 1 :

Profilage de l'expression des gènes d'*Azospirillum lipoferum* au cours de l'interaction avec le riz

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Cette partie fait l'objet d'un article soumis au journal

Molecular Plant-Microbe Interaction

Genome wide profiling of *Azospirillum lipoferum* gene expression during interaction with rice roots

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Abstract

Azospirillum-plant cooperation has been mainly studied from an agronomic point of view leading to a wide description of mechanisms implicated in plant growth-promoting effects. However, little is known about genetic determinants implicated in bacterial adaptation to the host plant during the transition from free-living to root-associated lifestyles. This study aims at characterizing global gene expression of *Azospirillum lipoferum* 4B during its interaction with two cultivars of *Oryza sativa* L. *japonica* (cv. Cigalon from which it was originally isolated, and cv. Nipponbare). Rice-associated *Azospirillum* cells are sessile but metabolically active, and gene expression is tightly adjusted to the host plant. Adaptation to rice involves genes related to detoxification of ROS and unknown plant compounds, as well as complex regulatory networks. As revealed by the induction of genes encoding insertion sequences, interaction with root may drive bacterial genome rearrangements. The complex regulation of genes implicated in the turnover of c-di-GMP secondary messenger suggests its involvement in *Azospirillum*-rice cooperation. Several genes display cultivar-specific expression profiles, highlighting host specific adaptation and raising the question of *A. lipoferum* 4B/rice cv. Cigalon co-adaptation.

Keywords: *Azospirillum*, Cooperation, Plant Growth-Promoting Rhizobacteria, Rice, Transcriptomic

II.1.1. Introduction

Rhizosphere constitutes an important microhabitat characterized by sustainable interactions between plants and rhizobacteria, which are essential for plant productivity. Plants exude up to 11% of fixed carbon via their roots, supporting rhizosphere microbial populations (Bais *et al.* 2006; Jones *et al.* 2009). In return, rhizobacteria provide nutrients to the plant and improve plant growth via specific mechanisms such as nitrogen fixation and phytohormone secretion (Richardson *et al.* 2009). These beneficial interactions between plant and bacteria involve rhizobial symbionts (mutualism) or plant growth-promoting rhizobacteria (PGPR, cooperation). Whereas mutualistic associations require partner recognition and a specific molecular crosstalk between plants and invading bacteria (Oldroyd *et al.* 2011), mechanisms involved in the cooperation (associative symbiosis) between PGPR and plants have been overlooked.

Among PGPR, members of the genus *Azospirillum* are known to colonize roots of important cereals and other grasses, and they constitute the dominant microorganism in rice rhizosphere (Lu *et al.* 2006; Steenhoudt and Vanderleyden 2000). Several *Azospirillum* strains exert phytostimulatory effects on plant growth and crop yields, and therefore constitute a promising alternative to reduce chemical inputs in the context of sustainable agriculture (Bashan *et al.* 2004). This plant growth-promoting effect was originally attributed to *Azospirillum* ability to fix atmospheric nitrogen, but the involvement of *Azospirillum* biological nitrogen fixation (BNF) in plant growth promotion is still debated (Bashan and de-Bashan 2010). It is well admitted that *Azospirillum* PGPR effect is mainly due to the production of several phytohormones allowing an increase in the number of lateral roots and root hairs, which results in higher nutrient and water uptake by the plant (Somers *et al.* 2004). Nevertheless, production of nitric oxide (NO) was also evidenced as strongly involved in the *Azospirillum*-induced root branching (Molina-Favero *et al.* 2007). Next to increasing the number of lateral roots and root hairs, *Azospirillum* also increases root exudation (Heulin *et al.* 1987) and modifies the chemical structure of root cell wall (El Zemrany *et al.* 2007). More recently, it was evidenced that the composition of plant secondary metabolites varies according to

Azospirillum strain/plant cultivar combinations, reviving the question of host specificity in phytostimulating rhizobacteria (Chamam *et al.* 2013; Drogue *et al.* 2012; Walker *et al.* 2011). When inoculated on two rice cultivars, *Azospirillum lipoferum* 4B displays a similar colonization pattern on both cultivars, but promotes plant growth and modifies secondary metabolic profiles more dramatically on its original cultivar Cigalon (Chamam *et al.* 2013).

A better understanding of the *Azospirillum*-plant interaction at the molecular level is essential to unravel mechanisms governing host specificity and to enable its rational use in biofertilization. So far, the molecular basis of *Azospirillum*-plant interactions has been investigated only by means of bacterial cultivation in presence of root exudates (Van Bastelaere *et al.* 1993, Pothier *et al.* 2007) or auxin indole-3-acetic acid (Van Puyvelde *et al.* 2011), but no global analysis was performed on cells grown directly in contact with the plant.

In this context, the current study aims at characterizing the molecular basis of the associative symbiosis between *A. lipoferum* 4B and two cultivars of *Oryza sativa* L. *japonica* (cv. Cigalon and cv. Nipponbare) using global gene expression analysis of *Azospirillum* root-associated cells. The model *A. lipoferum* 4B was originally isolated from the rhizosphere of *Oryza sativa japonica* cv. Cigalon in Camargue (Thomas-Bauzon *et al.* 1982) and its genome is available allowing transcriptome analysis (Wisniewski-Dyé *et al.* 2011).

II.1.2. Results and discussion

II.1.2.1. *Azospirillum* whole genome expression patterns

The aim of this study was to identify and distinguish *Azospirillum* genetic determinants particularly implicated in the transition from planktonic to sessile lifestyle from those directly implicated in the adaptation to the host plant. Thus, liquid cultures of *A. lipoferum* 4B in late-exponential phase (planktonic condition) were inoculated on roots of two rice cultivars (root-associated conditions) belonging to the Japonica group, cv. Cigalon and cv. Nipponbare, as well as on artificial root surfaces (sessile condition); root-adhering bacteria were recovered seven days after inoculation, a stage at which enhanced rice root growth is detectable (Chamam *et al.* 2013). At earlier stages, the setting did not allow the recovery of sufficient RNA amounts (necessary for transcriptome analysis), principally due to the small size of rice roots. The integration of two different growth conditions (i.e. planktonic condition and sessile condition) is thought to improve the identification of genes more likely involved in *Azospirillum*-rice interaction, as previously suggested by (Matilla *et al.* 2007).

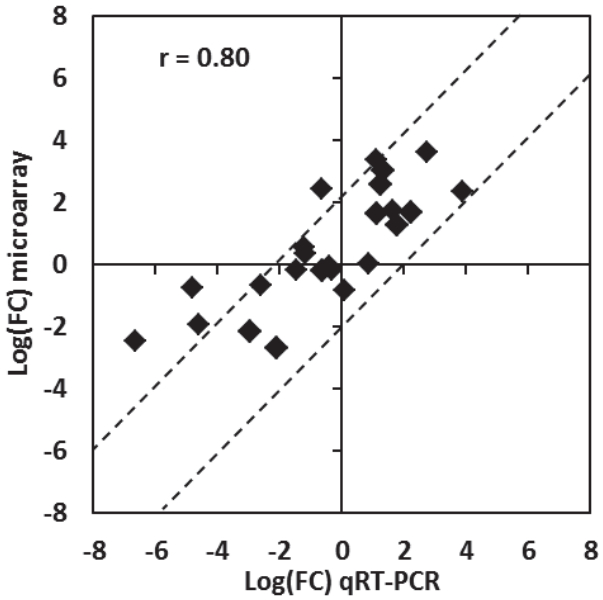


Figure 13 : Correlation of microarray and RT-qPCR results.
Expression ratios of 8 representative genes were determined using RT-qPCR, for planktonic condition (reference), sessile condition and root-associated conditions. Each microarray values (3 per gene; **Supplementary Table S2** *p96*) and RT-qPCR values (3 per gene; **Supplementary Table S2** *p96*) were log2 transformed and plotted against each other for comparison.

Hierarchical Clustering Analysis of microarray data reveals that root-associated conditions display the most dissimilar gene expression patterns compared to sessile and planktonic conditions (**Supplementary Data S1** *p95*). Genes differentially expressed were selected using an adjusted *P*-value (P_{adj}) threshold of 0.05 and a fold change (FC) cutoff of 2 (**Annexe III**). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to validate the microarray data. Expression ratios of eight representative genes obtained by RT-qPCR were plotted versus the respective microarray values showing that the RT-qPCR is in agreement with microarray data (**Figure 13** and **Supplementary Table S2** *p96*).

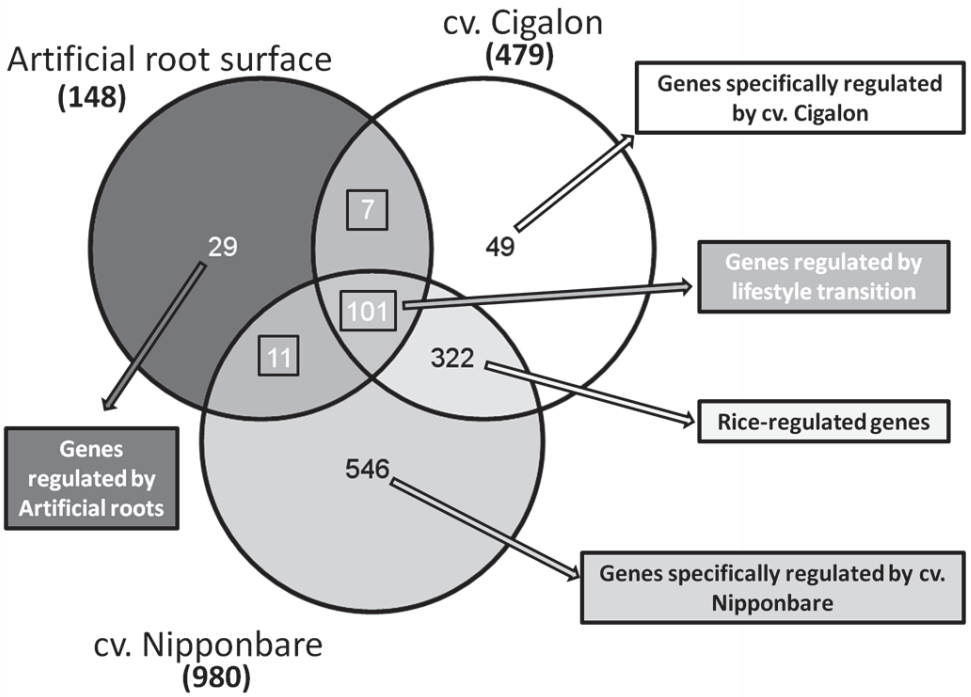


Figure 14 : Venn diagram representing differentially expressed genes (P_{adj}<0.05 and |FC|≥2).

Numbers in parentheses represent: the total number of regulated genes for each condition. Numbers in circles represent the number of genes for each comparison. Numbers in squares designate genes regulated by lifestyle transition.

A total of 148 genes are significantly regulated (P_{adj}<0.05 and |FC|≥2) for bacteria grown on artificial root surface, but mostly repressed (114 down-regulated, 34 up-regulated genes) (**Figure 14**, **AnnexeIII**). Interaction with rice roots modifies the expression of a wide number of bacterial genes that are mostly induced: 479 genes are significantly regulated for cv. Cigalon (252 down-regulated, 227 up-

regulated genes) and 980 for cv. Nipponbare (322 down-regulated, 658 up-regulated genes). Functional classification of *A. lipoferum* 4B genes significantly regulated in sessile and root-associated conditions reveals that major changes occur in the following functional classes: energy metabolism, protein synthesis, protein fate, transport and binding proteins, regulatory functions and signal transduction as well as cellular processes (**Figure 15**). As it was previously observed for *Pseudomonas putida* living in association with plant roots (Matilla *et al.* 2007), gene expression of rice-associated *Azospirillum* cells is tightly adjusted to the host plant.

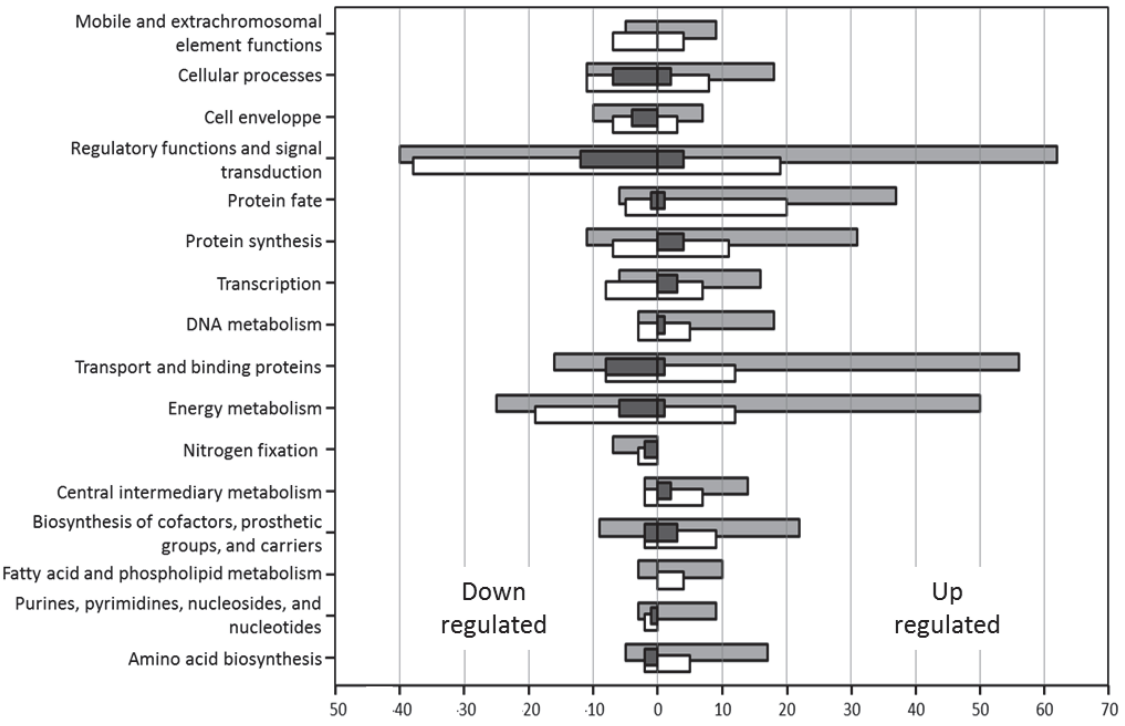


Figure 15 : Functional classification of *A. lipoferum* 4B differentially expressed genes.

Cells associated with cv. Cigalon (white), cv. Nipponbare (light grey) and "artificial roots" (dark grey) are compared to planktonic cells. Unclassified genes and genes of unknown function represent 48% of the 1065 differentially expressed genes (P_{adj} <0.05 and -2≥FC≥2). A full list of these genes is available in Supplementary Table S2. The functional analysis (Bioprocess) was done using the MaGe (Magnifying Genomes) interface (Vallenet *et al.* 2006) and available on the AzospirilluScope database (https://www.genoscope.cns.fr/agc/microscope/home/index.php).

Comparison of bacterial transcriptomes between sessile and root-associated conditions (with planktonic condition as the reference condition) unveils five categories of genes (**Figure 14**, **Annexe III**). The first category includes genes that are regulated only in cells associated with artificial-root (29) and will no longer be

discussed. The second category comprises 119 (101+7+11) genes whose expression is modified on both artificial and rice roots. Because these regulations occur when bacteria interact with both artificial and rice roots they cannot be considered as plant-related responses. However, it is noteworthy that most of these genes are down-regulated and involved in cell motility (*fliI1*, *flgB*, *flgF*, *cheY*) or membrane biosynthesis (*lpxC*, *lolD*, *mepA*, AZOLI_2268, AZOLI_1328), asserting a sessile status of *Azospirillum* cells during cooperation with rice (Whiteley *et al.* 2001).

The third category includes 322 genes (201 up-regulated, 121 down-regulated) whose expression is altered only when cells interact with rice roots of both cultivars (**Figure 14**; **Annexe III**). Those genes, mainly induced, are supposed to be involved in adaptation to rice. Almost one fifth of these genes are related to the following metabolic processes: i) amino acids biosynthesis, ii) biosynthesis of cofactors, iii) central intermediary metabolism, iv) energy metabolism, v) fatty acid and phospholipid metabolism, vi) nitrogen fixation, vii) protein synthesis and viii) nucleotides metabolism. Accordingly, *A. lipoferum* cells associated to rice roots are sessile but metabolically active, an observation previously made for *P. putida* colonizing maize roots (Matilla *et al.* 2007). Moreover, the establishment of a sustainable cooperation appears to depend on bacterial adaptation to plant mediated stresses as well as complex adjustments of regulatory networks, two features developed in the following sections. Surprisingly, genes involved in nitrogen fixation are mainly repressed suggesting that no significant BNF occurs in the tested conditions. However, the implication of BNF in plant growth improvements mediated by *Azospirillum* is still debated and growth promotion is supposed to be a combination of unrelated mechanisms (Bashan and de-Bashan 2010). *A. lipoferum* 4B genome harbors other genetic determinants potentially involved in plant-beneficial functions such as *acdS* encoding a protein involved in 1-aminocylcopropane-1-carboxylate (ACC) deamination and *nirK* encoding a protein involved nitric oxide production, that do not appear to be regulated in the tested conditions (Pothier *et al.* 2008; Prigent-Combaret *et al.* 2008; Wisniewski-Dyé *et al.* 2011). Considering stochastic events leading to cell-to-cell variations in bacterial mRNA levels and spatial variations in *Azospirillum* expression of functional genes along the root (Combes-Meynet *et al.* 2011, Raj and van Oudenaarden 2008, Vande

Broek *et al.* 1993), the regulation of beneficial properties and other key determinants could be underestimated in the present study.

The two remaining categories contain genes more specifically regulated by only one rice cultivar: cv. Cigalon or cv. Nipponbare (**Figure 14**; **Annexe II**). These results highlight the cultivar-specific adaptation of cooperative *Azospirillum* cells.

II.1.2.2. Adaptation to plant-mediated stresses

Adaptation to plant-mediated stresses is reflected by the up-regulation of genes implicated in reactive oxygen species (ROS) detoxification, heat shock response and genes encoding multidrug efflux (MDR) pumps, which occurs in cells associated with both cultivars (**Annexe III**). Several genes potentially implicated in DNA plasticity are also regulated pointing towards the implication of DNA rearrangements in adaptive events.

a. Detoxification of plant compounds

Several genes involved (or potentially involved) in ROS detoxification are up-regulated during *Azospirillum*-rice cooperation: *ohr* (encoding an organic hydroperoxide resistance protein), *sodB2* (encoding a superoxide dismutase), *cpo1* (encoding a non-heme chloroperoxidase), *trxA* (encoding a thioredoxin), *hybF* (involved in the maturation of hydrogenases 1 and 2) and two putative oxidoreductases (AZOLI_p30350 and AZOLI_p50438). In addition, genes potentially involved in cell damage repair caused by ROS are up-regulated. It includes *msrA* and *msrB* genes that encode a ubiquitous peptide methionine sulfoxide reductase known to be implicated in oxidized proteins repair mechanisms (Ezraty *et al.* 2005). Genes encoding Clp proteases and the TldD peptidase, implicated in protein fate and shown to be induced in response to hydrogen peroxide in *Bradyrhizobium japonicum* (Jeon *et al.* 2011), are also up-regulated in *A. lipoferum* 4B cells associated with rice roots.

The induction of several MDR efflux pumps of the RND (*acrA2*, AZOLI_p10652) and SMR (AZOLI_p50211) families indicates that ROS are not the only toxic agents encountered by *A. lipoferum* 4B root-associated cells. The induction

of the phage shock protein operon (*pspABC*) and of genes implicated in heat shock response (*hspD1*, *hspD2*, *dnaK, groEL1, groES1*) or universal stress response (AZOLI_2829), indicates that *A. lipoferum* 4B faces and adapts to diverse stress conditions. Moreover, exopolysaccharides constitute a potential protection against toxic compounds and two glycosyl transferases potentially implicated in exopolysaccharides biosynthesis (AZOLI_1257 and AZOLI_p60199) are up-regulated.

These results suggest that ROS detoxification and multidrug efflux are important features of *A. lipoferum* 4B cooperative cells and not only during the very early stages of root colonization. This is consistent with the fact that ROS are continuously produced in plant and that root exudates contain a large number of compounds that mediate positive and negative plant-bacteria interactions (Bais *et al.* 2006; Pauly *et al.* 2006). For example, plant-mediated stress could involve phenolic compounds exuded by rice roots, such as alkylresorcinols (Chamam *et al.*, in press). Indeed, these compounds were reported to induce *dnaK* in *Escherichia coli* biosensor (Miché *et al.* 2003), and this gene is also up-regulated in *A. lipoferum* 4B root-associated cells. Whereas the role of MDR pumps in bacteria has been mainly investigated in phytopathogens, some pieces of evidence tend to demonstrate their implication in beneficial bacteria-plant interactions (Matilla *et al.* 2007; Ramachandran *et al.* 2011). In particular, a RND-type efflux system was reported to play a host-specific role in the *Bradyrhizobium*-legume symbiosis (Lindemann *et al.* 2010). Plant-exuded metabolites were shown to regulate a wide range of *Azospirillum* genes, and some of them might induce both stress responses and signaling pathways (Drogue *et al.* in press; Pothier *et al.* 2007; Van Puyvelde *et al.* 2011). For example, ROS were suggested to play a role in signaling processes during bacteria-plant symbioses (Pauly *et al.* 2006; White and Torres 2010). As inoculation of *Azospirillum* modifies plant secondary metabolites profiles, and as secondary metabolites modulate gene expression of the bacterium (Chamam *et al.* 2013; Walker *et al.* 2010), *Azospirillum*-plant associations appear to involve a complex and reciprocal adaptation of both cooperative partners.

b. Genome rearrangements

Adaptation to the plant may also induce genome rearrangements in *Azospirillum* cells. Two copies of insertion sequences *ISAli3* and *ISAli9* are indeed up-regulated (AZOLI_0073, AZOLI_0093, AZOLI_0584 and AZOLI_1648) regardless of rice cultivar, indicating a potential enhanced transposition activity in root-associated cells. The genomic region encompassing the two induced *ISAli3* transposases (AZOLI_0073, AZOLI_0093) is flanked by a putative phage terminase (AZOLI_0047) and a putative phage integrase (AZOLI_0097); this region may have undergone multiple genomic rearrangements, partly due to transposases and phages activities. Moreover, genes encoding components of proteins involved in DNA amplification and repair, namely a subunit of excinuclease ABC (AZOLI_p30254) and the chi subunit of DNA polymerase III (*holC*), are up-regulated in root-associated cells. Taken together these results suggest that interaction with plants may drive genome plasticity of root-associated bacteria. Because genome rearrangements mediated by insertion sequences may lead to gene inactivation or neighbouring-gene regulation, transposition is generally maintained at low levels in bacterial cells (Mahillon and Chandler 1998). However, similar inductions suggesting DNA rearrangements were reported for *P. putida* in maize rhizosphere (Matilla *et al.* 2007). *In vitro,* several *Azospirillum* strains, including *A. lipoferum* 4B, display large-scale genomic rearrangements associated to phase variation; these events might occur in the rhizosphere as a non-swimming strain displaying all the features of the 4B variant has been isolated simultaneously and at the same frequency than strain 4B (Bally *et al.* 1983; Vial *et al.* 2006). Recent genomic analyses of *Azospirillum* revealed that most of the genes encoding critical functions for the association with plants were horizontally acquired (Wisniewski-Dyé *et al.* 2011). Thus, understanding whether DNA rearrangements are induced by a general stress response or a particular plant signal may unravel mechanisms leading to *Azospirillum* genome evolution.

II.1.2.3. Lifestyle transition and regulatory networks

Tight adjustments observed in *Azospirillum* root-associated cells involve complex regulatory networks; indeed a high number of genes implicated in

regulatory functions and signal transduction are regulated in cells interacting with rice roots of both cultivars (**Figure 15**; **Supplementary Table S2** *p96*).

Table V. Genes potentially involved in c-di-GMP turnover with significant regulation (P_{adj}<0.05) in root-associated *Azospirillum* cells.

Gene label	Fold Change[a]			Other domains identified
	Artificial root	cv. Cigalon	cv. Nipponbare	
Genes products containing a GGDEF domain[b] (PF00990)				
AZOLI_0003	ns	ns	2.12	response regulator receiver (PF00072)
AZOLI_0265	-2.80	-2.93	-3.05	response regulator receiver (PF00072)
AZOLI_0976	ns	ns	-1.31	response regulator receiver (PF00072) / PAS (PF13426)
AZOLI_2108	-1.62	-1.71	-1.73	response regulator receiver (PF00072)
AZOLI_2514	ns	ns	-1.51	response regulator receiver (PF00072)
AZOLI_p10650	ns	ns	3.32	None
AZOLI_p20268	ns	-4.25	-3.71	transmembrane region of the 5TM-Lyt (PF07694)
AZOLI_p20632	ns	ns	-1.66	PAS_9 (PF13426)
AZOLI_p30504	ns	ns	-1.36	bacterial extracellular solute-binding domain family_3 (PF00497)
Genes products containing an EAL domain (PF00563)				
AZOLI_1066	-2.91	-2.73	-1.64	None
AZOLI_2578	-9.08	-6.92	-6.43	None
AZOLI_p30532	ns	ns	-1.62	None
Genes products containing both GGDEF and EAL domains (PF00990 and PF00563)				
AZOLI_0179	ns	ns	-1.43	GAF_2 (PF13185)
AZOLI_2507	ns	-1.33	ns	None
AZOLI_p30269	ns	ns	-1.37	PAS (PF00989) / PAS_9 (PF13426)
Genes products containing a HD-GYP domain (PF01966)				
AZOLI_1508	ns	ns	2.41	RelA_SpoT (PF04607) / TGS (PF02824) / ACT_4 (PF13291)
AZOLI_1713	ns	ns	1.62	Nucleotidyltransferase (PF01909) / GlnD PII-uridylyltransferase (PF08335) / ACT (PF01842)

[a] ns, non significant = P_{adj}>0.05.
[b] Domains were identified according to the *Pfam* classification.

a. Signal Transduction

Among signal transduction systems described in prokaryotes (Galperin 2004; Ulrich and Zhulin 2010), the genome of *A. lipoferum* 4B contains 53 genes annotated as putative diguanylate cyclases and phosphodiesterases (or GGDEF- and EAL / HD-GYP-domain proteins) and 17 of them are regulated (P_{adj}<0.05) in root associated cells (**Table V**), suggesting the involvement of bis-(3',5')-cyclic-dimeric-guanosine

monophosphate (c-di-GMP) signaling in root-associated *Azospirillum*. Indeed, these proteins are known to be implicated in c-di-GMP turnover (Hengge 2009). C-di-GMP is an intracellular signaling molecule known to play a key role in motility and biofilm formation; high c-di-GMP levels were shown to stimulate biofilm-related functions and to repress motile activities (Hengge 2009). In *Azospirillum* root-associated cells, induction of the *psp* operon (mentioned above), some components of Clp proteases (mentioned above) and alleles of sigma factor RpoH (σ^{32}), as well as the repression of *laf1* gene, may indicate the formation of a mature biofilm and the inhibition of swarming motility (Beloin *et al.* 2004; O'Toole and Kolter 1998; Verstraeten *et al.* 2008; Whiteley *et al.* 2001). However, the complexity of c-di-GMP signaling has been underestimated. For example, contrary to the current paradigm some active diguanylate cyclases were evidenced to reduce early biofilm formation (Sanchez-Torres *et al.* 2012). In addition, mature biofilms are dynamic structures with bacterial cells harboring a myriad of different physiological states unlikely to follow a generic pre-determined developmental pathway (Patell *et al.* 2010, Mc Dougald *et al.* 2012), making the c-di-GMP signaling difficult to decipher. In this context, unraveling the complexity of c-di-GMP turnover during *Azospirillum*-rice cooperation could help understanding its adaptive significance.

In addition to c-di-GMP signaling, signal transduction involves histidine kinases (Ulrich and Zhulin 2010). In the current study, two genes annotated as sensor histidine kinases are regulated in both cv. Cigalon- and cv. Nipponbare-associated cells. The first, AZOLI_p40375 is down-regulated and displays 55% of identity with *Rhodosprillum centenum* RegB, part of the highly conserved redox-responding two-component system RegB/RegA (Elsen *et al.* 2004). However, the impact of AZOLI_p40375 repression on the subsequent signal transduction is difficult to apprehend, and the role of RegB/RegA system in *Azospirillum* interaction should be further investigated. The second, AZOLI_1541 is up-regulated (FC>8 in both conditions, up-regulation validated by RT-qPCR) (**Supplementary Table S2** *p96*). AZOLI_1541 is atypical as it harbors a receiver domain (hybrid histidine kinase) and contains no transmembrane domain; moreover the closest response regulator that might be coupled to AZOLI_1541 lies 10 kb downstream. Functional

analysis of AZOLI_1541 should be further undertaken in order to identify the role and the effector of this signal transduction system.

b. Plant-induced transcriptional regulation

A total of 31 transcriptional regulators are differentially expressed regardless of the rice cultivar (13 up-regulated and 18 down-regulated) and expression levels are validated by RT-qPCR for five of them (AZOLI_0326, AZOLI_1654, AZOLI_2103, AZOLI_p20158, AZOLI_p40490) (**Supplementary Table S2** *p96*). These results reflect the wide reorganization of *Azospirillum* physiology in root-associated cells. Several families are represented among the differentially expressed regulators (AraC, Crp/Fnr, GntR, MarR, MerR, OmpR, SirA, TetR, and XRE) suggesting that various signals are perceived by *Azospirillum* in the root micro-environment. The transcriptional regulator AZOLI_p10651 belonging to the TetR family and localized upstream of an up-regulated multidrug efflux pump (AZOLI_p10652 discussed above), is strongly up-regulated in response to both rice cultivars (Supplementary Table S1). Two additional TetR regulators (AZOLI_1411, AZOLI_2103) are also induced, which suggests that members of the TetR family, known to control genes involved in multidrug resistance, catabolic pathways, osmotic stress resistance and pathogenicity, play a key role in plant-bacterial signaling in the rhizosphere (Matilla *et al.* 2007; Ramos *et al.* 2005). Transcriptional regulators of the GntR family may also play a key role in *Azospirillum*-plant signaling, as revealed by the regulation of AZOBR_50003 in response to the presence of auxin (indole-3-acetic acid) in *Azospirillum brasilense* Sp245 cultures (Van Puyvelde *et al.* 2011).

Transcriptional regulation also involves the regulation of RNA polymerase sigma factors. *Azospirillum* strains (and more particularly *A. lipoferum* 4B) harbor a remarkably high number of *rpoH* paralogues. While most of the α-proteobacteria harbor two copies of genes encoding RpoH sigma factors, this gene is present in five copies in the strains *A. brasilense* Sp7 and *A. brasilense* Sp245 whereas six copies are found in *A. lipoferum* 4B genome (Kumar *et al.* 2012). Interestingly, five of these six copies are induced in rice-associated cells: whereas *rpoH4* and *rpoH6* genes are induced respectively three and ten-fold in both sessile and root-associated conditions, *rpoH1*, *rpoH3* and *rpoH5* are significantly induced only in root-associated

cells (14<FC<37), suggesting that *rpoH* alleles are finely regulated during the adaptation of *A. lipoferum* 4B to rice roots. Interestingly, the only allele of *rpoH* that does not appear to be regulated in any of our tested conditions is *rpoH2*, which was recently shown to control the photooxidative stress response in *A. brasilense* (Kumar *et al.*, 2012). In addition to the fine regulation of *rpoH* (σ^{32}) alleles, *rpoD* and two putative anti-sigma factors (AZOLI_0947, AZOLI_2510) are induced whereas two anti-sigma factors antagonists (AZOLI_0314, AZOLI_1029) are repressed during the interaction with rice roots. Sigma factors are known to play a key role in bacteria-plant beneficial interactions and particularly in the expression of beneficial properties of bacterial symbionts. Indeed, *Rhizobium* and *Sinorhizobium* mutated in *rpoH1* gene were affected in nitrogen fixation and nodules formation (Martinez-Salazar *et al.* 2009; Mitsui *et al.* 2004). Moreover *rpoH* was suggested to regulate auxin production in *Azospirillum brasilense* (Spaepen *et al.* 2007).

II.1.2.4. Evidence for host-specific adaptation in *Azospirillum*-rice cooperation

The composition and structure of PGPR communities are conditioned by plant genotypes and several lines of evidence are in favor of a genotype-specific adaptation of cooperative phytostimulating rhizobacteria (Drogue *et al.* 2012, Hartmann *et al.* 2008). Recently, plant secondary metabolite profiling evidenced the specific interaction between *A. lipoferum* 4B and its original host cultivar (cv. Cigalon) (Chamam *et al.* 2013). In this context, the fact that gene expression changes are of lower importance in *A. lipoferum* 4B cells associated with cv. Cigalon suggests that evolutionary processes could have led to a more specialized interaction (**Figure 14; 15**).

a. Regulated genes that are unique to *A. lipoferum* 4B

Among the 322 genes regulated regardless of the rice cultivar, 61 genes encode proteins with no orthologues in the three other genomes recently used to determine the *Azospirillum* core genome: *A. brasilense* Sp245 isolated from wheat, *A. brasilense* CBG497 isolated from maize and *Azospirillum* sp. B510 isolated as a rice endophyte (Kaneko *et al.* 2010, Wisniewski-Dyé *et al.* 2011, 2012 **Annexe I**) (**Supplementary Table S3** *p97*). While genes encoding proteins of unknown function represent about 45% of the 322 rice-regulated genes, they represent 86%

of 61 genes unique to *A. lipoferum* 4B. However, half of these genes display expression modification higher than five-fold (up or down) suggesting their essential role in cooperation of *A. lipoferum* 4B with rice roots. The fact that some *A. lipoferum* 4B unique genes are regulated during interaction with rice suggests that co-evolution could have shaped some key genetic determinants of the *A. lipoferum* 4B-rice interaction. These strongly regulated genetic determinants include genes encoding: PchR (transcriptional regulator of the AraC family), YoeB (toxin of the toxin/antitoxin YoeB/YefM system), a putative methyltransferase (AZOLI_p60129), a putative glycosyl transferase (AZOLI_p60199) and a putative excinuclease ABC (AZOLI_p30254). PchR is a transcriptional regulator of the pyochelin synthase (*pch*) operon. In the tested conditions, induction of *pchR* is not associated to significant induction of the *pch* operon. However, pyochelin is a siderophore shown to be implicated in the induction of systemic resistance against fungus pathogen in tomato and rice, so that its role in *A. lipoferum* 4B-rice interaction should be further investigated (Audenaert *et al.* 2002, De Vleesschauwer *et al.* 2006).

b. Cultivar-dependent regulated genes

While some bacterial genes are regulated regardless of the rice genotype, other key genetic determinants appear to vary in a cultivar dependent manner, as illustrated in Fig. 2. Indeed, 49 genes are significantly regulated only during the interaction with cv. Cigalon and 546 genes only with cv. Nipponbare (**Figure 14; Annexe III**). However, those numbers include genes with significant P_{adj} in both conditions that are discriminated only on the basis of small FC value differences between the cultivars. To exclude these genes and potential artifacts, only genes that are discriminated on the basis of both P_{adj} and FC ($P_{adj}<0.05$ and $|FC|\geq 2$ with one cultivar; $P_{adj}>0.05$ and $|FC|<2$ with the other cultivar) will be discussed in this section (**Annexe III**).

It represents 26 genes (of the 49 previously cited) that seem specifically regulated during interaction with cv. Cigalon and 344 genes (of the 546 previously cited) that seem specifically regulated by cv. Nipponbare. A vast majority of these genes are implicated in regulatory functions and signal transduction (**Annexe III**). Three putative sensor histidine kinases (AZOLI_1900, AZOLI_p20362 and

AZOLI_p30320) and a putative serine/threonine kinase (AZOLI_p40554) are up-regulated only with cv. Nipponbare. About 20 transcriptional regulators belonging to AraC, CopG, LuxR, TetR and Rrf2 families display cultivar-specific expression profiles. Cultivar-specific differences are also observed for genes implicated in amino acids, nucleotides and fatty acids metabolism as well as transport and binding proteins. In particular, 20 components of ABC transporters and at least two operons potentially implicated in amino acids transport (AZOLI_p10075-78 and AZOLI_p20684-86) seem preferentially regulated with cv. Nipponbare (**Figure 16**).

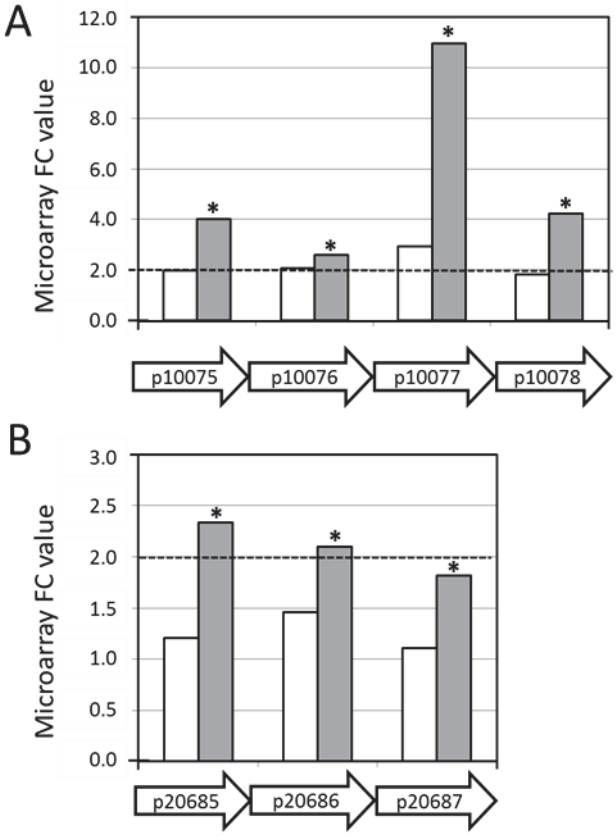


Figure16 : Microarray FC values of ABC transporter operons specifically regulated by cv. Nipponbare. **A**, Short-chain amide ABC transporter operon, **B**, Amino acid ABC transporter operon. White bars represent FC in *A. lipoferum* 4B cells associated with cv. Cigalon and grey bars represent FC values in *A. lipoferum* 4B cells associated with cv. Nipponbare. Dotted lines indicate FC cuttof of 2. Asterisks indicate significant values (P_{adj} <0.05). Genes are identified according to their label without the prefix AZOLI_.

In addition, *argF*, *argG*, *ocd* and *speF*, four genes involved in arginine and proline metabolism are induced only with cv. Nipponbare. These inductions are consistent with the presence of arginine and proline in root exudates of seven-day old rice plants but to our knowledge, differences in amino acids concentrations have never been investigated at the cultivar level (Bacilio-Jiménez *et al.* 2003). While exporters of plant toxic compounds were identified to be involved in *Rhizobium* adaptation to a specific plant species (Ramachandran *et al.* 2011), such related genes

are not evidenced among the *Azospirillum* cultivar-specific response. However, several genes encoding thioredoxin (*trxB*, *trxC*, AZOLI_p40170) or superoxide dismutase (AZOLI_1390) are specifically induced with cv. Nipponbare, suggesting that *A. lipoferum* 4B faces a more important oxidative stress when associated with this cultivar than with the one the strain has been isolated (cv. Cigalon).

As mentioned above, transcriptomic changes are of lower importance in bacterial cells associated with cv. Cigalon suggesting that plant cultivar/bacterial strain co-adaptation may have occurred, leading to a more specialized interaction. The hypothesis of co-adaptation of both partners can be supported by specific changes in root exudation, and root metabolites profiles induced on a strain dependent manner. Indeed, strains *A. lipoferum* 4B and *A. brasilense* A95 (isolated from rice, France) caused an increased rice exudation whereas *A. brasilense* R07 (isolated from rice, Senegal) and *A. lipoferum* B7C (isolated from maize) did not stimulate rice exudation, compared with sterile control (Heulin *et al.* 1987). Moreover, inoculation of *Azospirillum* on maize and rice induces modifications of secondary metabolite profile in both roots and shoots depending on *Azospirillum* strain/cultivar combinations, which suggests specific adaptation of the whole plant (Walker *et al.*, 2010; Chamam *et al.* 2013). Particularly, *A. lipoferum* 4B preferentially increased growth of the cv. Cigalon from which it was isolated and plant secondary metabolite profiling evidenced specific interaction between the strain and its original host cultivar (Chamam *et al.* 2013). Altogether, these results demonstrate that *Azospirillum*-rice cooperation involves wide and tight changes in both partners depending on strain/cultivar combinations.

II.1.3. Conclusion

Analysis of *A. lipoferum* 4B whole genome expression, realized on artificial root- and rice root-associated cells, highlights a tight adjustment of bacterial gene expression during *Azospirillum*-rice interaction (**Figure 17**). The establishment of a sustainable cooperation depends on bacterial adaptation to ROS and potentially plant-exuded phenolic compounds. As previously suggested, bacterial adaptation to the rhizosphere appears to involve genome rearrangements and c-di-GMP

secondary messenger turnover. *A. lipoferum* 4B adaptation to rice roots cooperation implicates a large number of transcriptional regulators and a remarkably high number of *rpoH* paralogues. Because the targets of 80% of these transcriptional regulators are unknown, a thorough investigation of *Azospirillum* regulatory systems seems an essential issue to better understand of *Azospirillum*-plant cooperation. The involvement of genes unique to *A. lipoferum* 4B and cultivar-specific regulation of genes implicated in amino acids metabolism raises the question of *A. lipoferum* 4B/rice cv. Cigalon co-adaptation.

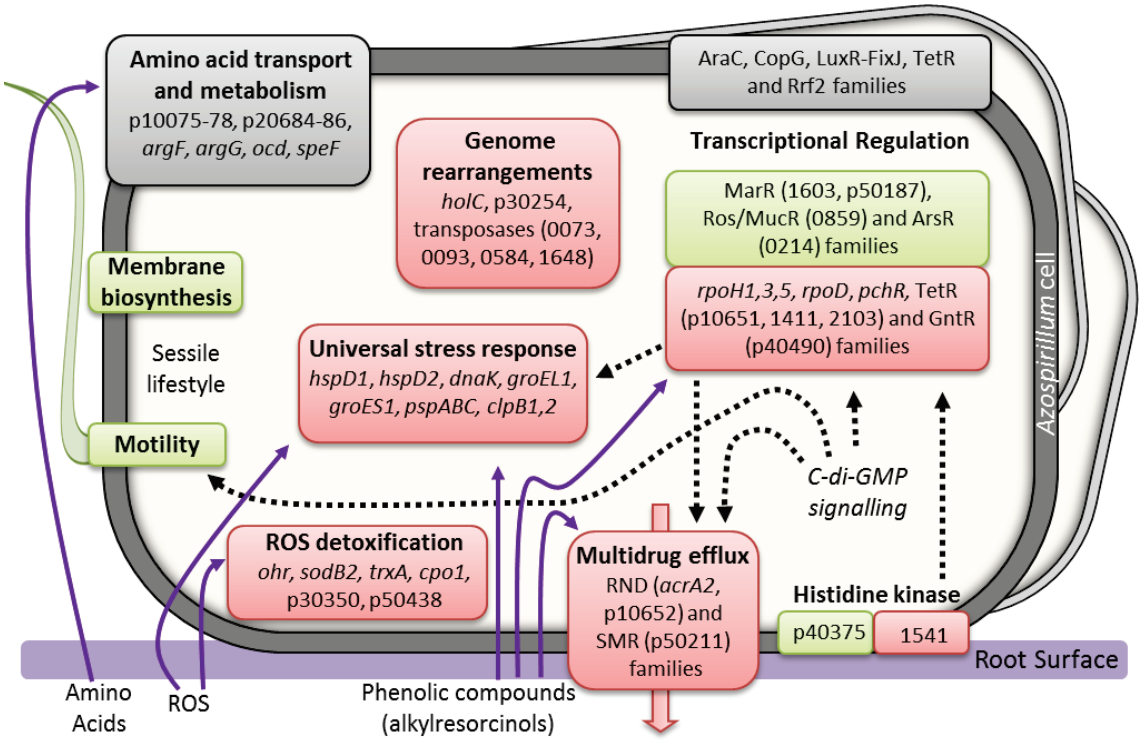


Figure 17. Overview of gene expression during *Azospirillum* adaptation to rice roots.
Up-regulated functions are highlighted in red and down-regulated functions in green. Functions displaying cultivar-specific responses are highlighted in grey. Black dotted arrows symbolize potential regulatory link between the functions. Purple arrows symbolize potential link between plant exuded compounds and regulated functions. Nameless genes are identified according to their label without the prefix AZOLI_.

II.1.4. Materials and methods

II.1.4.1. Bacterial strain and growth conditions

The plant growth promoting bacteria *A. lipoferum* 4B (Thomas-Bauzon *et al.* 1982) was grown overnight (180 rpm) at 28°C in nitrogen-free basal broth supplemented with 0.25% of LBm, i.e. Nfbm (Vial *et al.* 2006). Bacterial cells were harvested in the late-exponential phase, i.e. at OD580 around 1.2.

II.1.4.2. Seed sterilization, germination conditions, plant inoculation and plant growth conditions

Two rice (*Oryza sativa* L.) cultivars belonging to the Japonica group, cv. Cigalon (C. Louvel, Centre Français du Riz, Arles, France) and cv. Nipponbare (J.B. Morel, BGPI, Montpellier, France) were used for the study. Rice seeds were surface sterilized by washing for 40 min in a sodium hypochlorite solution containing 1 g of Na_2CO_3, 30 g of NaCl, and 1.5 g of NaOH per liter of distilled water (Hurek *et al.* 1994). Seeds were then rinsed 5 times for 3 min in demineralized sterile water, and chlorine traces were removed by washing 3 times for 7 min in sterile-filtered 2 % (w/v) sodium thiosulfate, and by rinsing 5 times for 3 min in demineralized sterile water (Miché and Balandreau 2001). Surface sterilized seeds were germinated on sterile plant agar (8 g l^{-1}) (Sigma Chemical Co, Saint Louis, USA) for 2 days in the dark at 28°C. A 10 ml aliquot of bacterial cells in late-exponential phase was transferred in a 50 ml BD Falcon™ tube (BD, Franklin Lakes, USA) for further RNA extraction (see below) and the rest was centrifuged, resuspended and introduced into 120 × 120 × 17 mm square plates as described by Chamam *et al.* (in press). For both rice cultivars, five disinfected seeds were laid onto the plates and 30 plates were realized. All the plates were incubated vertically, for 7 d in a growth chamber (MLR350, SANYO, UK) with a photoperiod of 16 h at 28°C (light 150 µE m^{-2} s^{-1}), and 8 h at 22°C in the dark. Two inoculations were performed independently.

II.1.4.3. Growth of bacteria on artificial root surfaces

In order to mimic root surface, four cellulose acetate filters (Satorius Stedim Biotech GmbH, Goettingen, Germany) were used as artificial root surfaces and placed onto 120 × 120 × 17 mm square plates (Greiner Bio-One Ltd., Stonehouse,

UK) containing 50 ml of Nfbm plant agar (8 g l^{-1}). 300 µl of bacterial cell suspensions used for plant inoculation, were inoculated on top of each filter. All the plates containing the inoculated artificial root surfaces were incubated in the same conditions as for plant growth experiments. Two inoculations were performed independently.

II.1.4.4. Bacterial cells isolation from planktonic, sessile and plant conditions

Four different conditions (two independent samples per condition) were used for the transcriptomic analysis, a planktonic condition as control, a sessile condition corresponding to the artificial root surface experiment and two plant conditions corresponding to inoculation of rice plantlets. For the planktonic condition, 20 ml of RNAprotect Bacteria Reagent (Qiagen, Courtaboeuf, France) were added to 10 ml of bacterial cells in late-exponential phase and centrifuged during 20 min, at 15°C, 10,000 rpm. The supernatant was discarded and the pellet was immediately frozen using liquid nitrogen and stored at -80°C. For the sessile and the plant conditions, 2×4 filters and 2×35 plant root systems were respectively pooled in two 50 ml BD Falcon™ tubes containing 8 ml of TE buffer and 16 ml of RNAprotect Bacteria Reagent (Qiagen). Bacterial cells were recovered by vortexing vigorously, 4 times for 1 min. For each condition, the two supernatants containing recovered bacteria from 4 filters or 35 plant root systems were pooled in a new 50 ml BD Falcon™ tube. The new tubes containing bacteria recovered from 8 filters or 70 plant root systems for each cultivar, were centrifuged during 20 min, at 15°C, 10,000 rpm. Supernatants were discarded and the pellets were immediately frozen using liquid nitrogen and stored at -80°C.

II.1.4.5. RNA isolation, amplification and cDNA synthesis

For each condition, the bacterial cell pellet was resuspended in 960 µl of suspension buffer (Prigent-Combaret *et al.* 2012) and transferred in 1.5 ml tubes containing 400 mg of glass beads (Sigma). Cell lysis was realized by shaking twice for 1 min with the TissueLyser II equipment (Qiagen); after a centrifugation of 5 min, at 4°C, 13,000 rpm, the aqueous phase containing ribonucleic acids was taken, and 1 ml of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was added. After incubation of 5 min at room temperature, 100 µl of phenol/chloroform/isoamyl

alcohol (25:24:1) were added, the samples were homogenized, incubated during 5 min at room temperature and centrifuged for 10 min, at 4°C, 13,000 rpm. A second phenol/chloroform/isoamyl alcohol extraction (200 µl) was done and ribonucleic acids were precipitated overnight at -20°C in a solution containing 2 volumes of 100 % ethanol, 0.1 volume of 7.5 M ammonium acetate and 0.01 volume of 5 mg ml^{-1} glycogen. Samples were centrifuged during 15 min, at 4°C, 13,000 rpm and the pellets were rinsed twice with 70 % ethanol before resuspension. PCR (16S *rrna*) on all samples confirmed that the DNase I (Invitrogen) treatment had removed all remaining DNA. RNA integrity was assessed using Agilent RNA 6000 Pico Kit (Agilent Technologies, Waldbronn, Germany) and the Agilent 2100 Bioanalyzer (Agilent Technologies) device.

In order to increase mRNA representation in RNA samples, total RNA were digested with mRNA ONLY™ Procaryotic mRNA isolation kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's protocol. The RNA samples were then amplified using MessageAmp™II-Bacteria Kit (Ambion Inc, Austin, TX, USA), with an amplification step of 6 h in order to obtain enough RNA for cDNA synthesis and to minimize amplification associated bias, according to Spiess *et al.* 2003.

The microarray cDNA was synthesized with the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen) following the provided protocol and using a mix (1:1) of random primers (Promega Corporation, Madison, WI, USA) and Oligo-dT(15) primers (Promega).

II.1.4.6. Microarray design, hybridization and data analysis

An *A. lipoferum* 4B whole genome expression array was designed by Roche Nimblegen, Inc. (Madison, WI, USA), based on the genome sequence (Wisniewski-Dyé *et al.* 2011), as follows: two replicates of 5 probes (length, 60 nucleotides) per gene, covering 6,242 genes and using a total of 62,178 probes. Each cDNA sample was labeled and hybridized by Roche Nimblegen according to their standard protocol. Two independent replicates were performed.

Data preprocessing and analysis was performed using Arraystar 4 software (DNASTAR, Inc., Madison, WI, USA) and the web available Analysis of NimbleGen Arrays Interface (ANAIS) (Simon and Biot 2010). The robust multi-array average (RMA) method associated with quantile normalization was applied at probe values (Irizarry *et al.* 2003, Bolstad *et al.* 2003). Probe values were summarized to gene values using median polish procedure. Analysis of variance with a false discovery rate adjustment method was applied (Benjamini and Hochberg 1995) and genes differentially expressed were selected using an adjusted *P*-value (P_{adj}) threshold of 0.05 and a two-fold cutoff for fold change (FC) (**Annexe III**). The planktonic condition was used as control.

The data are submitted in the National Center for Biotechnology Information Gene Expression Omnibus.

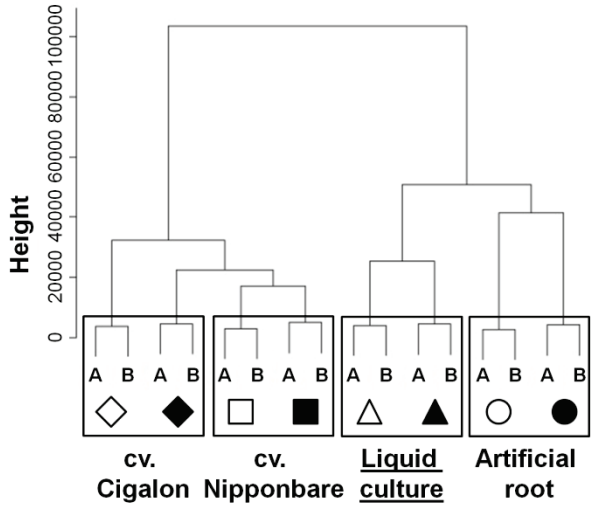
II.1.4.7. RT-qPCR

Gene expression level were validated by performing reverse transcription quantitative real-time PCR (RT-qPCR) on a group of 8 representative genes [Supplementary Table S2] using LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) on a LightCycler® 480 Real-Time PCR System (Roche). Two genes showing an invariant expression were used as reference genes: *uppS* (AZOLI_1074) and *nadE* (AZOLI_p30432) (**Supplementary Table S2** *p96*). Single strand cDNA was synthesized using the Omniscript® Reverse Transcription kit (Qiagen). An amount of 500 ng of total RNA used in the array experiment was incubated for 5 min at 65°C with 2 µl of random primers (Promega) in a final volume of 14.75 µl. A volume of 5.25 µl of a solution containing 2 µl of 10X Buffer, 2 µl of 5mM dNTPs, 1.25 µl of 10 units µl-1 RNase inhibitor, and 1 µl of 4 units µl-1 Omniscript Reverse Transcriptase, was added to each sample before incubating for 1 h at 37°C. From these reactions, 2 µl of cDNA were used as template for RT-qPCR reaction. DNA contamination was checked with reactions that lacked reverse transcriptase as negative controls. Specific primers were designed using the Primer3Plus interface (Untergasser *et al.* 2007) with the following criteria: product size ranges 150-250, primer size comprised between 20 and 21 bases, optimal primer Tm 60°C. Real time PCR conditions were: a denaturation stage of 10 min at

95°C; an amplification stage of 50 cycles of 30 s at 94°C, 15 s at 65°C and 15 s at 72°C; and a melting curve stage of 5 s at 95°C and 1 min at 65°C increased to 97°C with a ramp rate of 0.11°C s-1. All reactions were performed in three technical replicates and carried out in LightCycler 480 Multiwell plate 96 (Roche) with adhesive sealings foils (Roche) in a final volume of 20 µl containing 4 µl of nuclease free water, 2 µl of each primer (5 µM), 10 µl of master mix and 2 µl of the template. Primer efficiencies were determined by standard curves with serial dilution of DNA (5 $\log_{10}$ concentrations). The planktonic control was used as the calibrator condition and relative gene expression was calculated using the Pfaffl method (Pfaffl 2001).

Acknowledgements

We are grateful to C. Louvel (Centre français du riz, Arles, France) and J.B. Morel (BGPI, Montpellier, France) for gift of Cigalon and Nipponbare seeds respectively. We would like to thank A. Chamam for technical assistance. BD and SB received fellowships from the « Région Rhône-Alpes » and « Ministère de l'enseignement supérieur et de la recherche » respectively. This work was supported by the ANR project AZORIZ (ANR-08-BLAN-0098) and made use of the technical platforms "Serre" and DTAMB at FR41 (Université Lyon 1).



Supplementary Data S1. Hierarchical clustering analysis of *A. lipoferum* 4B gene expression data.
Triangles indicate gene expression data for the planktonic condition, circles for the sessile condition, squares for cv. Cigalon root-associated condition and diamonds for cv. Nipponbare root-associated condition. Black and white symbols indicate the two biological replicates. A and B letters indicate the two probe replicates per slide.

Supplementary Table S2. Validation of microarray data by RT-qPCR.

Gene	Primers	Description	cv. Cigalon				cv. Nipponbare				Sessile condition			
			RT-qPCR*			Micro array	RT-qPCR*			Micro array	RT-qPCR*			Micro array
			uppS	*nadE*	**Mean**		*uppS*	*nadE*	**Mean**		*uppS*	*nadE*	**Mean**	
AZOLI_p20158	F9065 GTCGCGCACACTTTATCTGTC F9066 GTCGAAAAATGCAGGTCTTTG	Regulatory protein PchR, AraC Family	2.18	2.60	**2.39**	**6.15**	0.38	0.9	**0.64**	**5.40**	0.53	1.09	**0.81**	**0.89**
AZOLI_p40490	F9079 GGCTGTTGGATGTCTTCGAT F9080 GATTGTCGGATTGCAGAAGAC	Transcriptional regulator, GntR Family	3.10	3.70	**3.4**	**2.46**	1.85	4.39	**3.12**	**3.38**	0.24	0.49	**0.36**	**0.90**
AZOLI_1541	F9071 TCATTCATCGCCTTCAGTCA F9072 GACAGGAAGAACAGGGCTTG	Hybrid histidine kinase	2.28	2.72	**2.5**	**8.24**	3.97	9.41	**6.69**	**12.2**	0.69	1.42	**1.06**	**0.57**
AZOLI_p40205	F8509 GCTGGTGCTGTCCTCCC F8510 CCTTGGTGATGGCGATGAT	Hybrid histidine kinase	1.04	0.24	**0.64**	**0.90**	0.1	0.23	**0.17**	**0.63**	0.50	1.01	**0.76**	**0.98**
AZOLI_1654	F9150 ATTCCGGTTCATTCCGACAT F9151 ACATCCTCGACCAGCAATTC	Putative transcriptional regulator, Crp/Fnr family	4.29	5.13	**4.71**	**3.27**	8.79	20.83	**14.81**	**5.17**	0.29	0.6	**0.45**	**1.30**
AZOLI_2103	F9156 GTGATCGGCTTCTATCTCAGC F9157 TTCGAGGATGTCGAGATGAGT	Putative transcriptional regulator, TetR Family	1.96	2.34	**2.15**	**3.16**	1.27	3.01	**2.14**	**10.47**	1.20	2.45	**1.82**	**1.02**
AZOLI_p20268	F9255 ATCCTGATGCTGGGAACGAT F9256 CGTCGTTGACCTCCTTGAAAT	Putative diguanylate cyclase, GGDEF domain	0.12	0.14	**0.13**	**0.23**	0.02	0.06	**0.04**	**0.26**	0.28	0.58	**0.43**	**1.50**
AZOLI_0326	F9267 ATCTCAGATCCCCGTCATCAT F9268 GGTGAGGTCGAGTTCCCAAT	Two-component response transcriptional regulator, OmpR family	0.21	0.25	**0.23**	**0.16**	0.01	0.01	**0.01**	**0.18**	0.02	0.05	**0.04**	**0.60**
Reference genes *														
AZOLI_1074	F8584 GATCAGCAATTTCCTGCTGTG F8585 AGATCGCGCTTGGTGAAAT	Undecaprenyl pyrophosphate synthetase												
AZOLI_p30432	F9103 CCAGATGTCCCACTACAATGTC F9104 CGAAATTTCCGTCTTCAGGAT	Glutamine-dependent NAD(+) synthetase												

**uppS* and *nadE* reference genes were used to normalise RT-qPCR results

Supplementary Table S3. *A. lipoferum* 4B rice regulated genes with no orthologues in others Azospirilla.

Gene_Name	Gene_Product
Down-regulated genes	
AZOLI_0018	protein of unknown function
AZOLI_2942	protein of unknown function
AZOLI_2154	protein of unknown function; putative coiled-coil and ATPase domains
AZOLI_0459	protein of unknown function
AZOLI_2631	exported protein of unknown function
AZOLI_1058	protein of unknown function
AZOLI_0019	protein of unknown function
AZOLI_p30119	protein of unknown function
AZOLI_2981	protein of unknown function
AZOLI_p30155	*yoeB*
AZOLI_p60213	protein of unknown function
AZOLI_p40048	protein of unknown function
AZOLI_1938	conserved protein of unknown function
AZOLI_p30110	conserved protein of unknown function
AZOLI_p50086	no homology to any previously reported sequences
AZOLI_0304	protein of unknown function
AZOLI_0909	partial transposase, IS256 family (N terminal part)
AZOLI_p40381	protein of unknown function
AZOLI_p30189	protein of unknown function
AZOLI_3130	protein of unknown function
AZOLI_p20690	Putative two-component response regulator, CheY-like (fragment)
AZOLI_p10421	protein of unknown function
AZOLI_2011	protein of unknown function
Up-regulated genes	
AZOLI_p60210	conserved protein of unknown function
AZOLI_p60199	putative glycosyl transferase, family 2
AZOLI_p10370	conserved protein of unknown function
AZOLI_1792	conserved protein of unknown function
AZOLI_2752	protein of unknown function
AZOLI_0692	conserved protein of unknown function
AZOLI_2410	protein of unknown function
AZOLI_2359	protein of unknown function
AZOLI_2779	conserved protein of unknown function
AZOLI_1716	protein of unknown function
AZOLI_p10797	protein of unknown function
AZOLI_2884	protein of unknown function
AZOLI_p10430	conserved membrane protein of unknown function
AZOLI_p30362	protein of unknown function
AZOLI_0994	protein of unknown function
AZOLI_p40438	membrane protein of unknown function
AZOLI_2356	protein of unknown function

AZOLI_0693	conserved protein of unknown function
AZOLI_p20655	putative transcriptional regulator
AZOLI_p40057	conserved membrane protein of unknown function; Lysine exporter domain
AZOLI_p10005	conserved protein of unknown function
AZOLI_p50388	protein of unknown function
AZOLI_p20158	*pchR*
AZOLI_p10003	protein of unknown function
AZOLI_2689	protein of unknown function
AZOLI_p20524	protein of unknown function
AZOLI_p30166	protein of unknown function
AZOLI_p50002	protein of unknown function
AZOLI_2767	protein of unknown function
AZOLI_0232	protein of unknown function
AZOLI_p60129	putative methyltransferase
AZOLI_p30199	conserved exported protein of unknown function
AZOLI_1044	protein of unknown function
AZOLI_p30254	putative excinuclease ABC, C subunit
AZOLI_1543	protein of unknown function
AZOLI_p20512	conserved exported protein of unknown function
AZOLI_p20525	protein of unknown function
AZOLI_0006	exported protein of unknown function

Partie 2 :

Profilage de l'expression des gènes d'*Azospirillum lipoferum* au cours de l'interaction avec le blé, le maïs et le riz

II.2.1. Introduction

Dans la partie précédente (**Chapitre II, Partie 1**), l'étude de l'expression des gènes de la souche *A. lipoferum* 4B au cours de la coopération avec le riz montre un ajustement fin du transcriptome bactérien. Une partie de cette réponse (322 gènes) intervient indépendamment du cultivar de riz auquel la bactérie est associée. C'est par exemple le cas de la détoxication des espèces réactives de l'oxygène (ROS), produites en continue par les plantes ou plus fortement en réponse à des stress biotiques (Pauly *et al.* 2006), et de l'implication du di-GMPc dans la transduction du signal. De même, un certain nombre de régulateurs transcriptionnels sont exprimés quelle que soit la combinaison *A. lipoferum* 4B/cultivar. Parmi les 322 gènes régulés indépendamment de la combinaison considérée, 61 sont absents du génome cœur d'*Azospirillum* (Wisniewski-Dyé *et al.* 2012). Dans la mesure où la moitié de ces gènes présente un niveau de régulation (FC) supérieur à 5, ils semblent jouer un rôle important dans l'adaptation d'*A. lipoferum* 4B aux racines de riz. Ces déterminants génétiques pourraient avoir été fixés par des mécanismes de coévolution entre les partenaires de l'interaction.

D'autres gènes semblent quant à eux régulés spécifiquement au contact d'un des deux cultivars. En effet, 26 gènes d'*A. lipoferum* 4B sont régulés spécifiquement lors de l'interaction avec le cultivar Cigalon et 345 gènes avec le cultivar Nipponbare. Parmi eux, une grande partie est impliquée dans le métabolisme des acides aminés dont la composition varie en fonction du cultivar de riz (Aulakh *et al.* 2001). Ces changements sont moins importants lorsque *A. lipoferum* 4B est associée à son cultivar d'origine (cv. Cigalon) ce qui laisse penser que des mécanismes de coadaptation auraient conduit à une interaction plus spécialisée. Cette hypothèse est en accord avec des observations récentes montrant qu'une association « privilégiée » pourrait s'établir entre la souche *A. lipoferum* 4B et son cultivar d'origine, aboutissant à un effet bénéfique plus important (Chamam *et al.* sous presse) (**Tableau IV** *p66*).

Dans ce contexte, le travail qui suit s'intéresse à la réponse hôte-spécifique à l'échelle des *Poaceae*. Pour cela, *A. lipoferum* 4B a été inoculée sur le riz, le maïs et le

blé. Dans un premier temps, nous discuterons des gènes régulés au contact des trois céréales afin de mettre en évidence des mécanismes impliqués dans l'adaptation quelle que soit la plante. Ces résultats seront comparés à ceux obtenus à l'échelle des deux cultivars de riz, afin de mettre en évidence des mécanismes généraux d'adaptation à la rhizosphère des *Poaceae*. Nous chercherons ensuite à déterminer si ces gènes appartiennent principalement au génome cœur d'*Azospirillum* ou s'il s'agit de gènes spécifiques de la souche *A. lipoferum* 4B. Enfin, nous discuterons des gènes régulés spécifiquement au contact de l'une des trois céréales.

II.2.2. Résultats et discussion

L'objectif de cette étude est de mettre en évidence les déterminants génétiques d'*Azospirillum* potentiellement impliqués dans l'adaptation à une céréale. Pour cela, des cultures liquides d'*A. lipoferum* 4B en fin de phase exponentielle ont été utilisées pour inoculer les racines de 3 céréales : le riz (4B_Riz), le blé (4B_Blé) et le maïs (4B_Maïs). Les bactéries associées aux racines ont été récupérées sept jours après inoculation.

Le choix d'inoculer des bactéries en fin de phase exponentielle a été guidé par le fait qu'à ce stade, les cellules d'*Azospirillum* ont accumulé du poly-β-hydroxybutyrate (réserve cellulaire) et des exopolysaccharides (agrégation cellulaire), deux facteurs qui augmentent la résistance aux stress, la survie et l'attachement des cellules (Kadouri *et al.* 2003 ; Bahat-Samet 2004).

II.2.2.1. Données brutes et normalisées, contrôle qualité

L'analyse transcriptomique a été réalisée par hybridation sur une puce d'expression couvrant 6242 gènes (pour un total de 62 128 sondes). Le contrôle qualité des données d'hybridation fournies par Roche-Nimblegen a été réalisé à l'aide des outils disponibles sur la plateforme internet ANAIS (http://anais.versailles.inra.fr). Ainsi, les données brutes et normalisées ont été analysées pour chacune des quatre conditions testées à savoir : la culture liquide utilisée pour l'inoculation, et les conditions bactéries associées aux racines, 4B_Riz, 4B_Blé et 4B_maïs.

L'analyse des données brutes montre que pour chacune des quatre conditions, la variabilité de l'intensité d'hybridation entre les deux répétitions biologiques indépendantes est faible. Ceci se traduit par une médiane et des quantiles relativement proches d'une répétition à l'autre, comme le montre la répartition des valeurs illustrée par les boîtes à moustaches de la **Figure 18, A**. Malgré tout, cette variabilité apparaît plus importante pour la condition 4B_Riz (**Figure 18, A**). De plus si l'intensité d'hybridation moyenne est comparable pour les conditions culture liquide, 4B_Riz et 4B_Maïs, celle-ci est plus faible pour la

condition 4B_Blé. Cette intensité plus faible pourrait s'expliquer par une différence de qualité des ARN utilisés pour la synthèse d'ADNc ; cependant plusieurs contrôles qualité ont été réalisés à chaque étape et aucun n'a révélé une moindre qualité des échantillons 4B_Blé. Il est important de noter que ces variations pourraient se traduire par une sous-évaluation du nombre de gènes significativement régulés dans les conditions 4B_Blé et 4B_Riz.

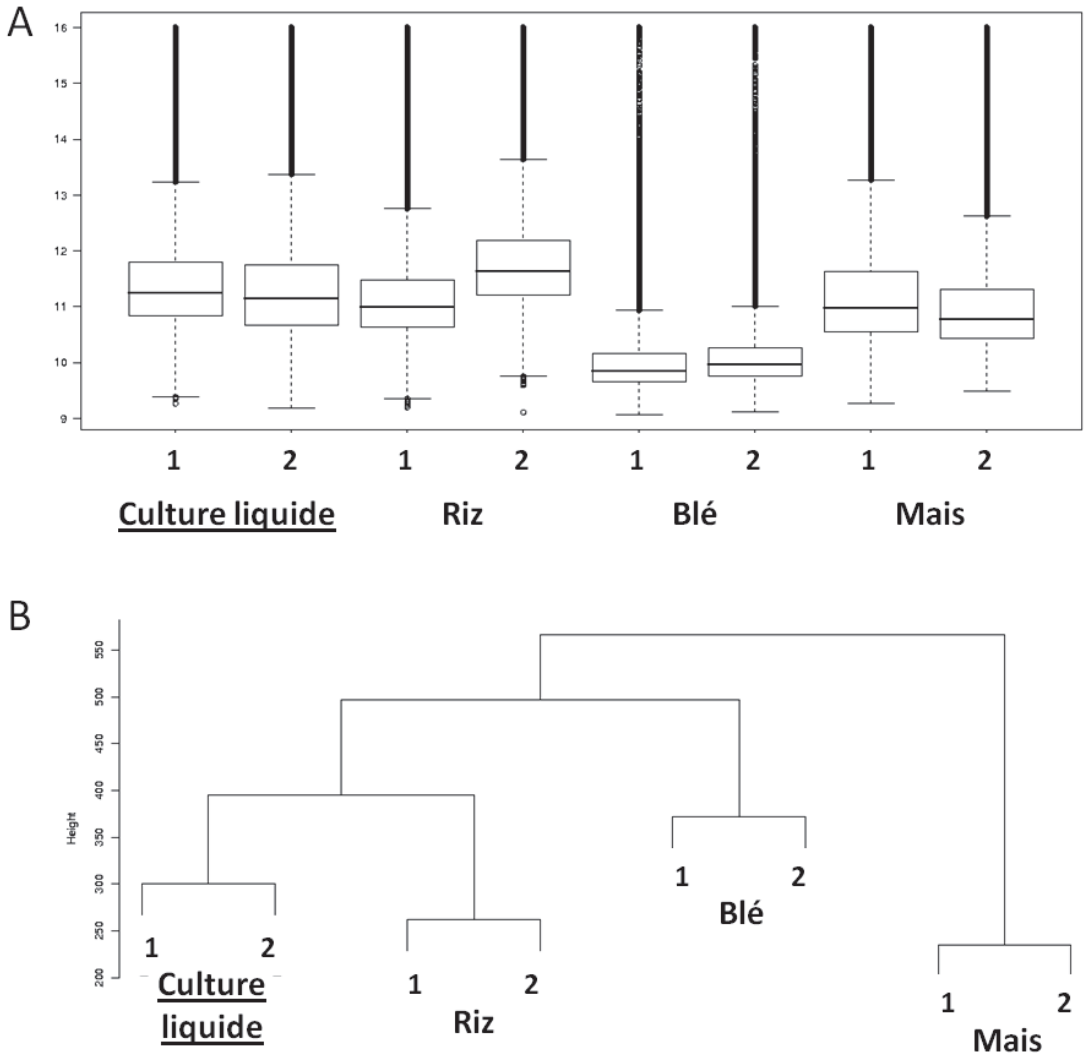


Figure 18 : Contrôle qualité des données d'hybridation.
A, Boîtes à moustaches représentant la répartition des intensités d'hybridation par sonde, réalisées à partir des données brutes pour chaque répétition (1,2) de chaque condition. B, Regroupement hiérarchique réalisé à partir des données normalisées pour chaque répétition (1,2) de chaque condition. Analyses réalisées à l'aide de la plateforme ANAIS (http://anais.versailles.inra.fr/) (Simon et Biot 2010).

L'analyse des données normalisées par la méthode RMA est conforme aux résultats attendus après normalisation. Ceci se traduit par une répartition uniforme des valeurs d'intensité d'hybridation, avec des médianes et des quantiles identiques pour les quatre conditions testées (Résultat non montré). Le regroupement hiérarchique de ces données à partir de la matrice des covariances montre que pour chacune des quatre conditions, les répétitions indépendantes se regroupent deux à deux (**Figure 18, B**). A l'inverse et conformément aux résultats attendus, les quatre conditions étudiées sont bien distinctes. Notons que la condition 4B_Maïs est celle qui présente le plus de différences avec la condition culture liquide. Ceci peut s'expliquer soit par le fait que les données 4B_Maïs étaient davantage homogènes avant normalisation, soit par des différences de régulation plus marquées. En tenant compte des limites expérimentales inhérentes au modèle d'étude qui conduisent à l'obtention d'une faible quantité d'ARN bactériens (et aux différentes adaptations techniques associées), la qualité des données paraît satisfaisante et compatible avec la poursuite des analyses.

II.2.2.2. Variation globale de l'expression des gènes d'*A. lipoferum* 4B

Les profils d'expression des gènes significativement régulés (P_{adj}<0,05 et |FC|>2) sont relativement hétérogènes d'une condition à l'autre (**Annexe IV**). En effet, 380 gènes sont régulés dans les bactéries associées aux racines de blé, majoritairement réprimés (80 induits contre 300 réprimés), alors que l'interaction avec les racines de maïs semble davantage induire l'expression des gènes bactériens, avec 986 gènes régulés dont 666 sont induits. Le statut des cellules bactériennes associées aux racines de riz est intermédiaire avec 493 gènes régulés et une proportion équivalente de gènes induits (234) et réprimés (259). La comparaison de ces profils d'expression a permis de mettre en évidence 7 catégories de gènes, illustrées par la **Figure 19**. Une catégorie regroupe 171 gènes régulés quelle que soit la condition. Trois catégories regroupent les gènes régulés spécifiquement dans une condition : 4B_Riz 65 gènes, 4B_Blé 116 gènes et 4B_Maïs 575 gènes. Les trois dernières catégories regroupent des gènes régulés dans seulement deux des trois conditions : 4B_Riz/4B_Blé 55 gènes, 4B_Blé/4B_Maïs 38 gènes et 4B_Riz/4B_Maïs 202 gènes.

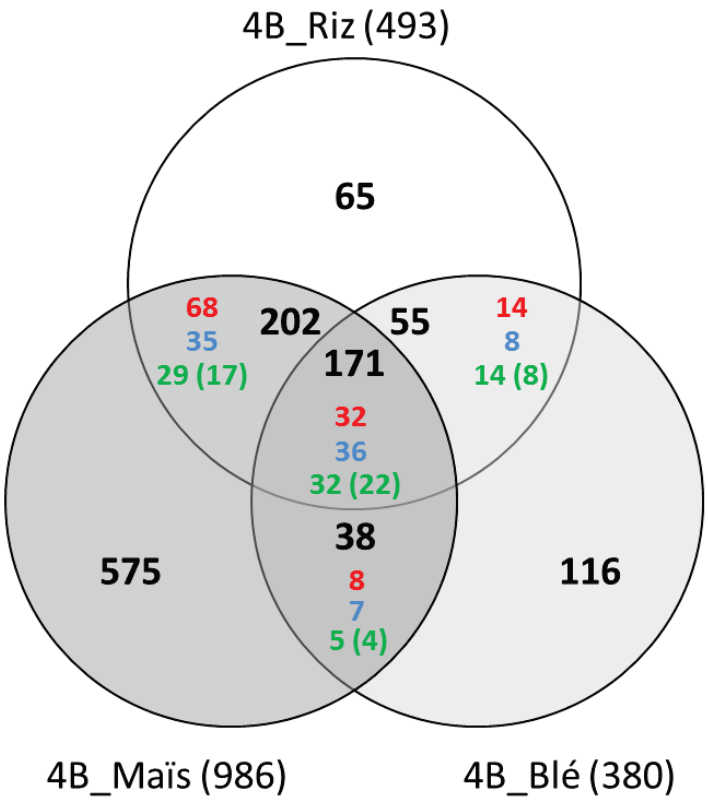


Figure 19 : Diagramme de Venn représentant les gènes significativement régulés (P_{adj}<0,05 et |FC|>2).
Noir : nombre de gènes régulés pour chaque comparaison. **Noir entre parenthèses** : nombre total de gènes régulés pour chacune des conditions. **Rouge :** nombre de gènes régulés appartenant au génome cœur d'*Azospirillum* et partagés avec le proche voisin phylogénétique *Rhodospirillum centenum* SW (ANC_Core ; **Annexe IV**) (Wisniewski-Dyé *et al.* 2012, **Annexe II**). **Bleu :** nombre de gènes régulés appartenant au génome cœur d'*Azospirillum* et absents du génome de *R. centenum* SW (AZO_Core absents de ANC_Core ; **Annexe IV**). **Vert** : gènes régulés absents des trois autres souches d'*Azospirillum* dont le génome entier est disponible. **Vert entre parenthèses** : nombre de gènes régulés pour lesquels aucun orthologue n'est trouvé dans l'ensemble des génomes séquencés et disponibles. La figure illustre le nombre de gènes régulés quelle que soit la condition, le nombre de gènes régulés spécifiquement dans une condition (4B_Riz, 4B_Blé ou 4B_Maïs) et le nombre de gènes régulés dans seulement deux conditions (4B_Riz/4B_Blé, 4B_Blé/4B_Maïs, 4B_Riz/4B_Maïs).

Ces résultats, comme ceux précédemment décrits (**Chapitre II, Partie 1**), témoignent d'une adaptation plante-spécifique de la bactérie. Les interactions avec le maïs et le riz semblent induire l'expression d'une proportion plus importante de gènes que l'interaction avec le blé. En effet, seulement 80 gènes sont induits dans la condition 4B_Blé. Notons qu'il s'agit de la condition présentant le plus faible niveau d'hybridation sur la puce et que le nombre de gènes induits pourrait être sous-estimé. Les résultats observés pour les trois plantes seront validés en réalisant des PCR quantitatives sur un nombre important de transcrits. Nous envisageons également des validations *in planta* à l'aide de fusions transcriptionnelles. Il sera

nécessaire de déterminer si les particularités du profil 4B_Blé sont d'origine biologique ou technique, et éventuellement envisager l'hybridation de nouveaux échantillons. Dans la mesure où la sous-estimation du nombre de gènes induits pour la condition 4B_Blé, pourrait conduire à une sous-estimation du nombre de gènes induits dans les trois conditions, nous distinguerons seulement deux grandes catégories de gènes dans la suite de ce travail : les gènes régulés dans au moins deux des trois conditions (466 gènes) et les gènes régulés spécifiquement dans une condition (756 gènes).

II.2.2.3. Adaptation aux céréales

Comme décrit plus haut, 466 gènes (171+202+55+38) sont régulés dans au moins deux des trois conditions. Parmi eux, 274 gènes avaient été détectés comme régulés indépendamment du cultivar de riz et 104 autres avaient été associés au style de vie sessile dans la précédente partie (**Chapitre II, Partie 1 ; AnnexeIV**). De manière générale, l'interaction entre *A. lipoferum* 4B et les céréales entraîne la répression de gènes impliqués dans la biosynthèse des membranes (*lolD*, *mepA*, *lpxC*), la mobilité (*fliI*, *flgB*) et le chimiotactisme (*cheY*). Ces résultats confirment que les cellules d'*A. lipoferum* 4B associées aux racines sont sessiles et se divisent peu, comme cela a été observé pour la bactérie *Pseudomonas putida* associée aux racines de maïs (Matilla *et al.* 2007 ; **Chapitre II, Partie 1**). L'induction de gènes impliqués (ou potentiellement) dans la détoxication des ROS (*ohr, sodB2, trxA, hybF*) et la réparation des dommages cellulaires (*msrA, msrB, clpA, clpB, tldD*) confirme l'importance de l'adaptation au stress oxydatif dans l'interaction *Azospirillum*-céréales. Cette réponse semble moins marquée pour les cellules associées aux racines de blé et le niveau d'induction est parfois très différent d'une condition à l'autre. Ceci pourrait s'expliquer par des différences dans la quantité de ROS produite par chacune des trois céréales. A notre connaissance, aucune étude comparative n'a évalué la production de ROS en réponse à l'inoculation d'*Azospirillum* chez ces trois céréales. Cependant, une étude menée sur deux génotypes de blé a mis en évidence des différences de concentration en H_2O_2 et une variabilité de l'activité des superoxides dismutases entre les génotypes en réponse à un stress salin (Sairam *et al.* 2002). Ainsi, des différences entre les trois céréales utilisées dans notre étude ne sont pas exclues.

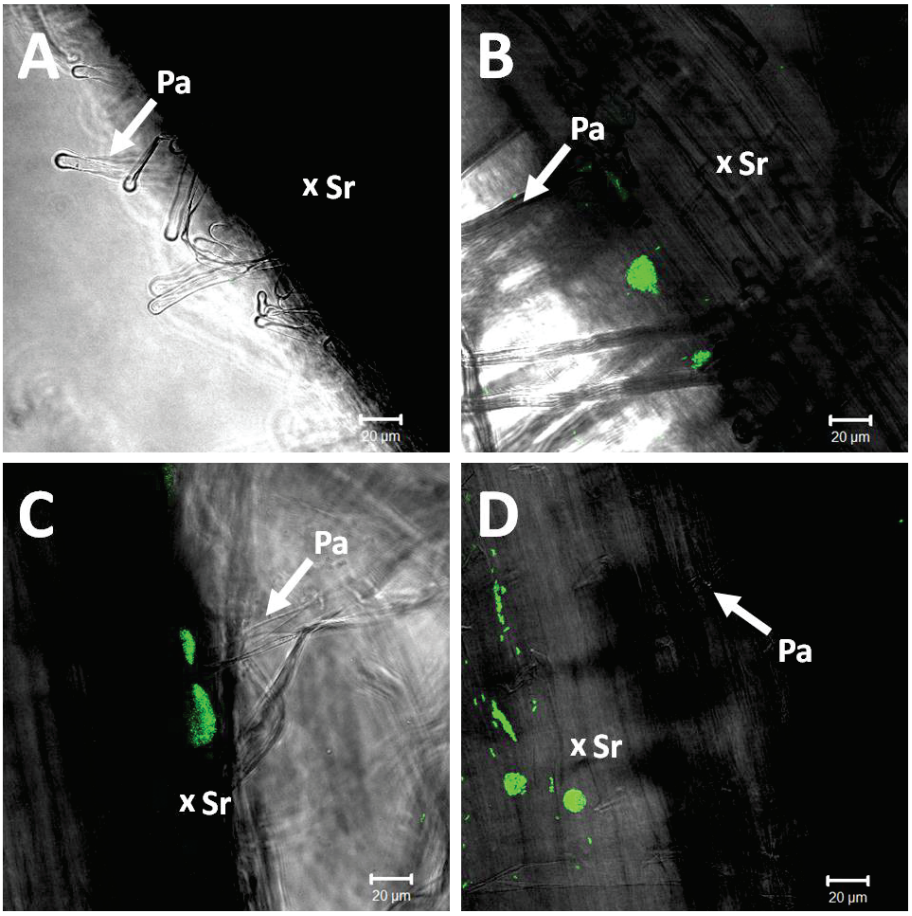


Figure 20 : Colonisation de la zone pilifère du riz, du blé et du maïs par *A. lipoferum* 4B observée en microscopie confocale à balayage laser.
A, racine de riz non inoculée. B, C, D, racines inoculées avec la souche *A. lipoferum* 4B pMP2444 (P*lac-egfp*). B, riz. C, blé. D, maïs. Les bactéries sous forme de micro-colonies apparaissent vertes sur les racines grises. Pa, Poil absorbant. Sr, Surface racinaire.

La variabilité observée entre les conditions pourrait également s'expliquer par des modes de colonisation racinaire de la souche *A. lipoferum* 4B différents pour chacune des trois céréales. En effet, de nombreuses études mettent en évidence l'hétérogénéité spatiale de l'expression des gènes d'*Azospirillum* sur les racines (Combes-Meynet *et al.* 2011, Vande Broek *et al.* 1993). De plus, plusieurs souches d'*Azospirillum* ont été décrites comme endophytes (Elbeltagy *et al.* 2001 ; Rothballer *et al.* 2003), un style de vie qui pourrait modifier la réponse oxydative du partenaire végétal. Si les études précédemment réalisées dans l'équipe ont montré que la souche *A. lipoferum* 4B colonise uniquement la surface des racines de riz (Chamam *et al.* sous presse), principalement dans la zone pilifère, aucune information n'est disponible pour cette souche sur le maïs et le blé. C'est pourquoi nous avons étudié le patron de colonisation d'*A. lipoferum* 4B sur les trois plantes 7 jours après inoculation. Les résultats illustrés par la **Figure 20** montrent que ce patron ne varie pas d'une plante à l'autre. En effet, les zones colonisées sont les mêmes quelle que soit la plante et le mode de colonisation est similaire. Ainsi, la zone apicale est faiblement colonisée par des cellules individuelles (résultats non montrés), alors que de nombreuses micro-colonies sont observées au niveau de la zone pilifère, et ce pour chacune des trois céréales. Seule la surface des racines est colonisée par la souche *A. lipoferum* 4B confirmant qu'il ne s'agit pas d'une souche endophyte des céréales.

L'étude des 10 gènes les plus fortement régulés pour chacune des trois conditions (**Tableau VI**), permet de mettre en évidence deux gènes fortement régulés au contact des trois plantes : AZOLI_0501 et AZOLI_p10651 régulateur de la famille TetR décrit précédemment (**Chapitre II, Partie 1**). Un orthologue du gène AZOLI_0501 est présent sur le chromosome des autres souches d'*Azospirillum* séquencées à ce jour, dans une région qui semble conservée au sein du genre *Azospirillum*. Ce gène code une protéine d'une taille de 70 acides aminés présentant un domaine retrouvé dans de nombreuses protéines de fonction inconnue (pfam06568). Un gène codant une protéine homologue (PP5274, 35 % d'identité) est retrouvé dans le génome de *P. putida* KT2440 ; ce gène est induit (FC=2,1) dans la rhizosphère du maïs (Matilla *et al.* 2007).

Tableau VI : Liste des 10 gènes les plus fortement induits pour chacune des trois conditions, classés selon leur FC.

Etiquette	FC 4B_Riz	FC 4B_Blé	FC 4B_Maïs	Gène	Produit
Riz					
AZOLI_p10651¥	89,61	21,42	119,21		putative transcriptional regulator, TetR family
AZOLI_0006‡	61,39	18,76	23,67		exported protein of unknown function
AZOLI_0501¥	60,17	6,90	43,24		protein of unknown function
AZOLI_p40308	55,85	4,66	21,53		protein of unknown function
AZOLI_1927‡	51,94	3,41	30,65		conserved membrane protein of unknown function
AZOLI_1830	50,47	4,26	16,03		membrane protein of unknown function
AZOLI_p40128	50,06	3,19	24,59		conserved protein of unknown function
AZOLI_0242	44,31	1,97	9,73	*pspB*	transcriptional regulator of *psp* operon
AZOLI_p30017	31,55	0,96	5,62	*hybF*	maturation of hydrogenases 1 and 2
AZOLI_p30180	28,61	2,68	16,75	*nhaA1*	sodium-proton antiporter
Blé					
AZOLI_p10651¥	89,61	21,42	119,21		putative transcriptional regulator, TetR family
AZOLI_p50338	1,83	19,01	2,45	*cyoC*	cytochrome *o* ubiquinol oxidase subunit III
AZOLI_0006‡	61,39	18,76	23,67		exported protein of unknown function
AZOLI_p10867	1,00	11,96	1,72		glycine betaine ABC transporter
AZOLI_p40055	14,32	7,81	25,26	*rpoH1*	RNA polymerase sigma factor (sigma32)
AZOLI_p10003*	6,80	7,16	7,73		protein of unknown function
AZOLI_0501¥	60,17	6,90	43,24		protein of unknown function
AZOLI_p50002*	8,18	6,68	18,54		protein of unknown function
AZOLI_0445	4,32	6,07	7,88	*rplK*	50S ribosomal subunit protein L11
AZOLI_1956	26,46	5,91	14,27		exported protein of unknown function
Maïs					
AZOLI_p10651¥	89,61	21,42	119,21		putative transcriptional regulator, TetR family
AZOLI_p20513	22,01	5,51	53,58		conserved protein of unknown function
AZOLI_p20512	18,15	4,77	48,85		conserved exported protein of unknown function
AZOLI_0501¥	60,17	6,90	43,24		protein of unknown function
AZOLI_p30049	19,23	1,96	42,20		putative transcriptional regulator MarR family
AZOLI_p20540	18,92	3,23	36,85	*rpoH5*	RNA polymerase sigma factor (sigma32)
AZOLI_p40454	14,03	2,89	32,24	*rpoH3*	RNA polymerase sigma factor (sigma32)
AZOLI_1927‡	51,94	3,41	30,65		conserved membrane protein of unknown function
AZOLI_p30260	8,32	3,97	26,97		putative FeS cluster assembly protein
AZOLI_2652	18,44	3,86	26,47	*furA2*	Ferric uptake regulator

¥ gène parmi les 10 plus induits dans les trois conditions

‡ gène parmi les 10 plus induits dans deux des trois conditions

Rouge : gènes appartenant au génome cœur d'Azospirillum et partagés avec le proche voisin phylogénétique *Rhodospirillum centenum* SW (ANC_Core) (Wisniewski-Dyé *et al.* 2012, **Annexe II**).

Bleu : gènes appartenant au génome cœur d'*Azospirillum* et absents du génome de *R. centenum* SW (AZO_Core absents de ANC_Core).

Vert : gènes absents des trois autres souches d'*Azospirillum* dont le génome entier est disponible.

* gènes pour lesquels aucun orthologue n'est trouvé dans l'ensemble des génomes séquencés et disponibles

Le régulateur de la famille TetR est situé en amont de deux gènes codant des pompes à efflux de type Emr-*like* (AZOLI_p10652 et AZOLI_p10653) (**Figure 21**), dont le premier est significativement induit en présence du riz et du maïs. Pour la condition 4B_Blé, l'induction d'AZOLI_p10652 n'est pas significative, mais la valeur de FC proche de deux suggère également une surexpression du gène. Il est intéressant de noter que plus le FC observé pour le régulateur est important plus le FC observé pour la cible potentielle AZOLI_p10652 est élevé (1,94 pour 4B_Blé, 4,99 pour 4B_Riz et 19,78 pour 4B_Maïs). Ces résultats suggèrent une corrélation entre l'expression du régulateur et celle du gène en aval. L'étude de la synténie montre que l'organisation de ces gènes est maintenue au sein du genre *Azospirillum* et plus généralement au sein des alpha-protéobactéries, associées ou non aux plantes (**Figure 21**).

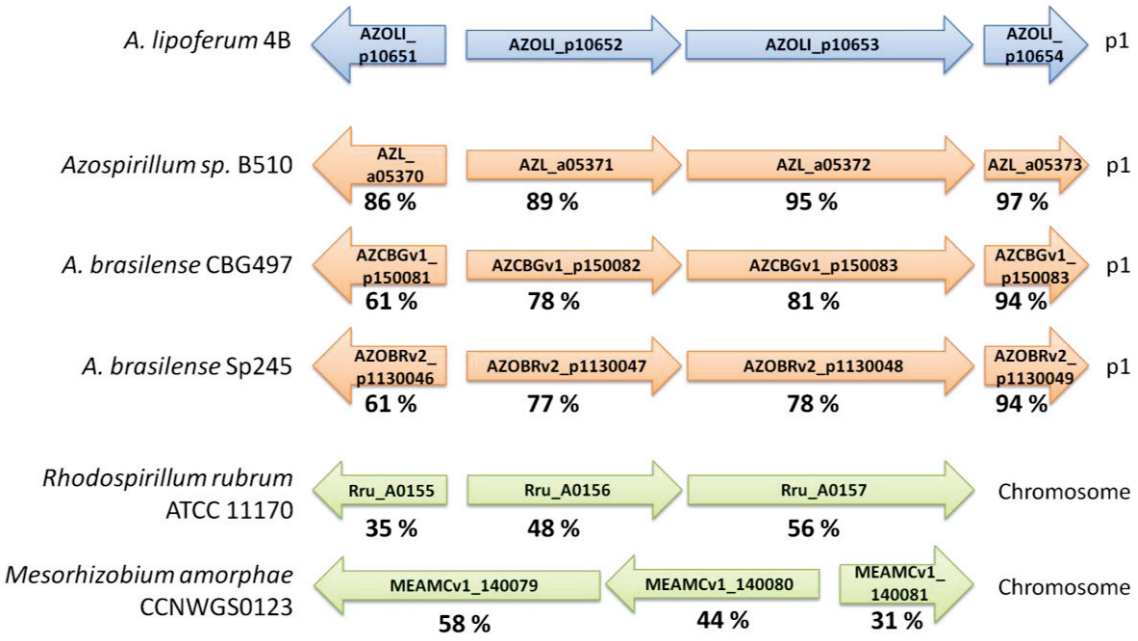


Figure 21 : Synténie des gènes AZOLI_p10651, AZOLI_p10652 et AZOLI_p10653 entre *A. lipoferum* 4B et d'autres α-protéobactéries.
Synténie adaptée à partir des informations sur le syntonome disponibles sur la plateforme MAGE (https://www.genoscope.cns.fr/agc/microscope/home/index.php). Les pourcentages indiquent l'identité des séquences protéiques de chacune des souches avec les séquences de la souche *A. lipoferum* 4B. p1, Plasmide p1.

Notons que chez les bactéries du genre *Azospirillum*, un gène additionnel dont la fonction est inconnue (AZOLI_p10654) est situé en aval de cette région. Il présente la particularité d'être fortement conservé avec un pourcentage d'identité entre les souches compris entre 94 à 97 %. L'ensemble de ces résultats sont en

faveur d'un rôle important des gènes AZOLI_p10651, AZOLI_p10652, AZOLI_p10653. Ils pourraient notamment être impliqués dans la résistance aux composés toxiques exsudés par la plante. En effet, chez la bactérie phytopathogène *Erwinia chrysanthemi*, l'inactivation du système EmrAB (33 % d'identité avec AZOLI_p10652) induit une sensibilité à divers antibiotiques ainsi qu'une inhibition totale de la croissance en présence d'extraits protéiques de pomme de terre (Valecillos *et al.* 2006). La mutation entraine également une diminution significative de la virulence sur les tubercules de pomme de terre. Chez *Rhizobium etli*, les mutants *rmrA* (gène codant pour une pompe à efflux de type Emr, 45 % d'identité protéique avec AZOLI_p10652) présentent des seuils de tolérance à la naringénine, à l'acide coumarique et à l'acide salicylique, plus faibles que la souche sauvage (González-Pasayo et Martínez-Romero 2000). De plus, ces mutants sont affectés dans la formation des nodosités (moins 40%). Cette perte de résistance à de fortes concentrations en naringénine peut être complémentée par les gènes *emrAB* de la bactérie *E. coli*. Ces résultats sont en faveur d'une similarité fonctionnelle entre les systèmes RmrAB et EmrAB (González-Pasayo et Martínez-Romero 2000) et soulignent l'importance des pompes à efflux dans l'adaptation à la plante hôte.

D'autres gènes sont fortement régulés dans les trois conditions, parmi lesquels deux gènes codant des protéines excrétées de fonction inconnue, AZOLI_0006 et AZOLI_1927. Ces gènes sont présents chez toutes les souches d'*Azospirillum* dont le génome est disponible. Le gène AZOLI_0006 code une protéine possèdant un motif LTXXQ (PF07813) retrouvé chez la protéine CpxP connue pour son rôle dans la réponse au stress alcalin. L'étude de la synténie montre que ce gène et les deux gènes en amont (AZOLI_0004 et AZOLI_0005) sont présents chez plusieurs souches du genre *Thiomonas*. Le gène AZOLI_1927 code une protéine possédant deux copies d'un domaine conservé de fonction inconnue (PF03733) retrouvé chez des protéines supposées membranaires.

Enfin, les gènes codant les régulateurs transcriptionnels RpoH font également partie des gènes fortement induits dans les trois conditions. Parmi les 6 copies du gène *rpoH* présentes dans le génome d'*A. lipoferum* 4B, les copies *rpoH1*, *rpoH3* et *rpoH5* apparaissent fortement surexprimées au contact des trois plantes alors que

les copies *rpoH4* et *rpoH6* sont plus faiblement induites dans les traitements 4B_riz et 4B_maïs. Au contact du blé, ces deux dernières copies ne sont pas significativement régulées.

II.2.2.4. Génome cœur d'*Azospirillum* et interaction avec la plante.

Les approches de génomique comparative récemment réalisées sur les génomes d'*A. lipoferum* 4B, *A. brasilense* Sp245, *A. brasilense* CBG497 et *Azospirillum sp.* B510 ont permis de définir le génome cœur d'*Azospirillum* (AZO-core) qui comporte 2328 gènes (Wisniewski-Dyé *et al.* 2012) **(Annexe II)**. Ainsi, le génome cœur décrit comme ancestral (ANC_core), c'est-à-dire partagé avec le proche voisin phylogénétique *Rhodospirillum centenum* SW, a également été défini (1121 gènes parmi les 2328 de AZO-core).

Sur la base de ces données, nous avons considéré l'origine des gènes régulés au contact d'au moins deux des trois plantes. Les analyses mettent en évidence que 208 des 466 gènes régulés dans au moins deux des trois conditions appartiennent au génome cœur d'*Azospirillum* (**Figure 19 ; Annexe IV**). Ces gènes sont particulièrement intéressants car ils représentent des fonctions communes aux quatre souches d'*Azospirillum* jouant un rôle dans l'adaptation aux céréales. Au-delà des gènes impliqués dans le fonctionnement de la chaîne respiratoire, le métabolisme énergétique et la traduction qui sont partagés avec le proche voisin phylogénétique *R. centenum* SW (122 des 208 gènes appartiennent à l'ANC_core), on retrouve plusieurs gènes absents du génome de *R. centenum* SW (**Annexe IV**). Ces gènes, au nombre de 86, pourraient avoir contribué de manière importante à l'établissement d'*Azospirillum* dans la rhizosphère au cours de la transition entre la vie marine et la vie terrestre décrite par Wisniewski-Dyé *et al.* (2011). En effet, on trouve parmi eux trois gènes impliqués dans des systèmes à deux composants (AZOLI_0326, AZOLI_0984, AZOLI_p40375), un gène codant un régulateur transcriptionnel de la famille MarR (AZOLI_p30049), deux gènes codant des ABC transporteurs (AZOLI_1291, AZOLI_p40473), un gène codant une perméase potentiellement impliquée dans le transport de composés toxiques (AZOLI_p50211) ainsi qu'un gène codant une pompe à efflux (AZOLI_p10652), discuté dans la section précédente. Plusieurs études ont montré le rôle essentiel des fonctions de transport

dans l'adaptation à la rhizosphère, soulignant l'importance d'étudier le rôle de ces gènes dans la coopération (Matilla *et al.* 2007 ; Ramachandran *et al.* 2011).

Nous avons également considéré les gènes retrouvés chez la souche *A. lipoferum* 4B, absents des trois autres souches séquencées. Ainsi, 80 des 466 gènes régulés dans au moins deux des trois conditions sont présents uniquement chez *A. lipoferum* 4B (**AnnexeIV**). Parmi eux, 51 gènes ne présentent aucun homologue dans l'ensemble des génomes entièrement séquencés et disponibles. Les 29 gènes restant présentent au moins un homologue dans les génomes des bactéries n'appartenant pas au genre *Azospirillum*. Ces 80 gènes pourraient être associés à des propriétés spécifiques d'une souche, exercées sur un large spectre de céréales comme par exemple le patron de colonisation des racines. En effet, nous avons montré que la souche *A. lipoferum* 4B colonise les trois plantes sous forme de micro-colonies à la surface des racines dans la zone pilifère (**Figure 20**). Or, comme nous l'avons détaillé dans le **Chapitre I**, **Partie 2** (**Fig.2.** *p40*), ce patron est différent de celui observé pour *A. brasilense* Sp245 qui colonise préférentiellement les poils absorbants quelle que soit la plante. Dans la mesure où 84% des gènes spécifiques cités précédemment ont une fonction inconnue, il reste difficile d'associer les caractéristiques écologiques des souches aux déterminants génétiques qui leurs sont propres. Cependant, ces déterminants apparaissent comme des candidats intéressants pour réaliser des analyses fonctionnelles.

II.2.2.5. Adaptation hôte-spécifique.

Comme dans le **Chapitre II**, **Partie 1**, afin d'éliminer les potentiels artéfacts dans la catégorie des gènes spécifiques de l'interaction d'*A. lipoferum* 4B avec une seule des céréales étudiées, nous avons choisi de prendre en compte uniquement les gènes pour lesquels la P_{adj} et le FC sont significatifs dans une condition et non significatifs dans les deux autres (par exemple, un gène est considéré spécifique de la condition 4B_Riz si : $P_{adj}<0.05$ et $|FC|\geq 2$ pour 4B_Riz alors que $P_{adj}>0.05$ et $|FC|<2$ pour 4B_Blé et 4B_Maïs) (**Annexe IV**).

En conséquence, les gènes spécifiques représentent 38 % des 1222 gènes régulés dans cette étude répartis comme suit : 21 gènes spécifiques de 4B_Riz, 58

gènes spécifiques de 4B_Blé et 381 gènes spécifiques de 4B_Maïs. Comme dans le cas de la comparaison entre les deux cultivars de riz (**Chapitre II, Partie 1**), de nombreux gènes codant des ABC transporteurs ou des pompes à efflux présentent un profil d'expression plante-spécifique. Ces résultats soulignent l'importance des mécanismes impliqués dans l'adaptation d'*A. lipoferum* 4B à la composition chimique des exsudats tant du point de vue de la protection contre les composés toxiques que de l'assimilation des nutriments. Notons que l'adaptation des systèmes de transport d'*A. lipoferum* 4B est marquée aussi bien à l'échelle interspécifique (entre le blé, le maïs et le riz) qu'à l'échelle intraspécifique (entre le cultivar Cigalon et le cultivar Nipponbare) soulignant la variabilité de la composition des exsudats mise en évidence à ces deux échelles (Aulakh *et al.* 2001 ; Bacilio-Jiménez *et al.* 2003 ; Czarnota *et al.* 2003 ; Fan *et al.* 2001). Si le nombre de gènes codant des transporteurs est, d'une façon générale, proportionnel à la taille des génomes bactériens, une sur-représentation de ces gènes a été décrite chez les alpha-protéobactéries associées aux plantes (Konstantinidis et Tiedje 2003). Ainsi, ce caractère est partagé avec des symbiotes mutualistes comme *Mesorhizobium loti* et *Sinorhizobium meliloti* (Konstantinidis et Tiedje 2003), dont la spécificité d'hôte est plus marquée que dans le cas des symbioses associatives. Ces observations suggèrent que la diversité des gènes codant les transporteurs est liée à l'avantage compétitif que procure cette propriété dans la rhizosphère. Dans la mesure où ils présentent des profils d'expression plante-spécifiques, leur impact sur la compétitivité des souches va dépendre du génotype végétal. Par exemple, chez *Rhizobium leguminosarum* l'inactivation du gène RL0680, codant un transporteur induit spécifiquement dans la rhizosphère du pois, réduit la compétitivité de la souche sur le pois mais pas sur la luzerne (Ramachandran *et al.* 2011).

Comme observé dans le **Chapitre II**, **Partie 1**, les gènes impliqués dans le métabolisme des acides aminés (et leur transport), présentent des profils d'expression plante-spécifiques. C'est notamment le cas de plusieurs gènes impliqués dans le métabolisme de la cystéine et de la méthionine (*cysk1, metY2* et *metZ*) qui sont induits uniquement dans la condition 4B_Maïs (**Annexe IV**). De nombreux gènes codant des régulateurs transcriptionnels sont également régulés spécifiquement au contact d'une des trois céréales ; c'est le cas du gène codant le

régulateur PchR (AZOLI_p20158) induit uniquement dans la condition 4B_Riz quel que soit le cultivar (**Chapitre II**, **Partie 1**) et qui présente la particularité d'être absent des trois autres souches d'*Azospirillum* dont le génome est entièrement séquencé et disponible. Ce régulateur contrôlant la synthèse de pyochéline, un sidérophore capable d'induire l'ISR chez le riz (De Vleesschauwer *et al.* 2006), les mécanismes évolutifs auraient pu conduire à la sélection de cette propriété chez les souches d'*Azospirillum* associées au riz. C'est pourquoi nous envisageons d'évaluer l'occurrence des gènes *pch* au sein du genre *Azospirillum*, par exemple en réalisant des PCR ou des Southern Blot. Il apparaît intéressant de savoir si ce gène est présent plus spécifiquement chez l'espèce *A. lipoferum*, ou chez la souche 4B ou plus généralement chez des souches d'*Azospirillum* ayant été isolées du riz. En parallèle, la construction d'un mutant *Azospirillum* incapable de synthétiser la pyochéline pourrait permettre d'étudier son impact sur la réponse immunitaire de la plante au cours de la coopération *A. lipoferum* 4B-riz.

II.2.3. Conclusion.

Cette étude a permis de mettre en évidence un ensemble de 466 gènes régulés au contact d'au moins deux des trois céréales étudiées : le blé, le maïs et le riz. Les gènes impliqués dans la mobilité, le chimiotactisme et la biosynthèse des membranes sont réprimés, confirmant le statut sessile des cellules d'*Azospirillum* associées aux racines. A l'inverse, un grand nombre de gènes impliqués dans les mécanismes de réponse au stress oxydatif sont induits suggérant un rôle important des ROS dans l'interaction *Azospirillum*-céréale. Parmi les gènes les plus fortement induits se trouvent un régulateur de la famille TetR, une protéine conservée de fonction inconnue ainsi que trois des six paralogues du gène *rpoH* présents dans le génome d'*A. lipoferum* 4B. Parmi ces 466 gènes, 208 appartiennent au génome cœur d'*Azospirillum* dont 122 sont partagés avec le proche voisin phylogénétique *R. centenum* SW. D'autres gènes (86) sont retrouvés uniquement chez la souche *A. lipoferum* 4B et pourraient être associés à des propriétés spécifiques d'une souche, exercées sur un large spectre d'hôtes, comme par exemple le patron de colonisation.

D'autre part, nous avons mis en évidence que certains mécanismes

d'adaptation dépendent de la combinaison *A. lipoferum* 4B / céréale considérée. Ainsi, 460 gènes présentent un profil d'expression plante-spécifique parmi lesquels de nombreux gènes sont impliqués dans le transport des nutriments et l'efflux de composés toxiques. La combinaison des résultats de génomique comparative et de transcriptomique permet de mettre en évidence des déterminants génétiques souche-spécifique régulés de manière plante-spécifique, comme le régulateur transcriptionnel PchR. A notre connaissance, cette approche n'a jamais été envisagée pour les interactions PGPR-plante et sa poursuite nous paraît particulièrement intéressante pour comprendre les mécanismes impliqués dans la spécificité d'hôte.

II.2.4. Matériel et Méthodes

II.2.4.1. Souches bactériennes et conditions de cultures.

La bactérie *A. lipoferum* 4B (Thomas-Bauzon *et al.* 1982) a été cultivée sur la nuit (180 rpm) à 28°C dans le milieu Nfbm (Vial *et al.* 2006). Les cellules bactériennes ont été récupérées en fin de phase exponentielle (à une DO_{580} proche de 1,2).

II.2.4.2. Stérilisation des graines, conditions de germination, inoculation des plantes et conditions de croissance.

Dans cette étude, des graines de riz (*Oryza sativa* L. Japonica) du cultivar Cigalon (Centre Français du Riz, France), de maïs (*Zea mays* L.) du cultivar PR37Y15 (Pioneer Semences, Aussonne, France) et de blé (*Triticum aestivum* L.) du cultivar Soissons ont été utilisées. Les graines ont été stérilisées comme décrit précédemment (**Chapitre II, Partie 1** *p90*) à l'aide d'une solution d'hypochlorite de sodium contenant du Na_2CO_3 (1g l^{-1}), du NaOH (1,5g l^{-1}) et du NaCl (30g l^{-1}) (Hurek *et al.* 1994). Pour les graines de blé, la désinfection a été précédée par un bain de 12h dans une solution aqueuse de Bénomyl (0,5mg ml^{-1}) contenant du Diméthylsulfoxide (5% V/V), afin d'éliminer les contaminations fongiques. Les graines stérilisées ont été déposées sur des boîtes d'eau gélosée (8g l^{-1} de Plant Agar, Sigma Chemical Co, Saint Louis, Etats-Unis) et incubées 48h dans le noir à 28°C pour germination. Les graines de riz et de blé ont été inoculées comme détaillé dans la **Partie 1** du **Chapitre II** (*p90*). Pour ces deux plantes, 30 boîtes contenant chacune 5

graines ont été préparées. Afin de s'adapter à la taille des plantules de maïs sans modifier les conditions d'interaction, un système 4 fois plus grand a été utilisé. Ainsi, 4.10^9 cellules bactériennes ont été mélangées à 200 ml d'eau gélosée (8g l^{-1}). Ce mélange a été déposé dans des boîtes stériles carrées (245 x 245 x 25mm). Pour le maïs, 4 boîtes contenant chacune 4 plantes ont été réalisées. Pour chacune des trois céréales, les boîtes ont été incubées 7 j dans des chambres climatiques (MLR350, SANYO, Royaume-Uni) avec une photopériode de 16h à 28°C à la lumière (150µE m^{-2} s^{-1}), et 8h à 22°C dans le noir. Deux inoculations indépendantes ont été réalisées pour chaque plante.

II.2.4.3. Récupération des cellules bactériennes à partir des cultures liquides et des racines inoculées.

Quatre conditions (deux échantillons indépendants par condition) ont été utilisées : le témoin bactérie planctonique correspondant à la culture liquide utilisée pour l'inoculation, et trois conditions bactéries associées aux plantes, une avec le riz, l'autre avec le blé et la dernière avec le maïs. Pour le témoin bactérie planctonique, 20 ml de RNAprotect Bacteria Reagent (Qiagen, Courtaboeuf, France) ont été ajoutés aux 10 ml de culture bactérienne en fin de phase exponentielle. Après centrifugation, le surnageant a été éliminé et le culot bactérien congelé dans l'azote liquide et stocké à -80°C (voir **Chapitre II, Partie 1** *p91*). Pour les bactéries associées au riz et au blé, 2x35 systèmes racinaires ont été rassemblés pour chaque céréale dans deux tubes BD Falcon™ de 50 ml contenant 8ml de tampon TE et 16ml de RNAprotect Bacteria Reagent (Qiagen). Pour le maïs, seulement 2x4 systèmes ont été rassemblés. Pour chaque céréale, les deux tubes contenant les systèmes racinaires ont été vortexés pendant 1 min à quatre reprises. Pour chaque type de plante, les deux surnageants contenant les bactéries détachées des systèmes racinaires ont été mis en commun dans un nouveau tube BD Falcon™ 50ml. Ainsi, pour le riz et le blé chaque nouveau tube contient les bactéries détachées de 70 systèmes racinaires ; pour le maïs, ce nouveau tube contient les bactéries détachées de 8 systèmes racinaires. Ces tubes ont été centrifugés 20 min à 15°C, 10 000 rpm. Les surnageants ont été éliminés et les culots congelés dans l'azote liquide et conservés à -80°C.

II.2.4.4. Extraction et amplification des ARN, synthèse des ADN complémentaires (ADNc).

Pour chacun des échantillons, l'extraction et l'amplification des ARN ainsi que la synthèse d'ADNc double brin ont été réalisées en suivant la méthode détaillée dans la **Partie 1** du **Chapitre II** (*p91*).

II.2.4.5. Caractéristiques de la puce d'expression, hybridation et analyse des données.

La puce d'expression d'*A. lipoferum* 4B a été dessinée par Roche Nimblegen, Inc. (Madison, WI, Etats-Unis) sur la base de la séquence du génome publiée par Wisniewski-Dyé *et al.* 2011. Elle comporte 5 sondes (60 nucléotides) par gènes et 2 réplicats par sonde, couvre 6242 gènes (sur 6354) pour un total de 62 128 sondes. Pour chaque condition les ADNc des deux répétitions indépendantes ont été marqués et hybridés par Roche Nimblegen selon leur protocole standard sur la puce d'expression décrite dans la **Partie 1** du **Chapitre I** (*p92*).

L'analyse des données a été réalisée à l'aide du logiciel Arraystar 4 (DNASTAR, Inc., Madison, WI, Etats-Unis) et de l'interface ANAIS disponible sur internet (Simon et Biot 2010), comme décrit précédemment (**Chapitre I**, **Partie 1**) (*p92*). Les gènes sont considérés comme significativement régulés si la probabilité critique (P_{adj}) est inférieure à 0,05 et un niveau d'expression relatif (FC) supérieur à 2 ou inférieur à -2. La culture liquide (bactérie planctonique) a été utilisée comme référence.

II.2.4.6. Analyse du patron de colonisation d'*A. lipoferum 4B* par microscopie confocale à balayage Laser.

Le patron de colonisation de la souche *A. lipoferum* 4B a été analysé sur le riz, le maïs et le blé. Pour cela, la souche marquée avec le plasmide pMP2444 P*lac-egfp* (Chamam *et al.* sous presse) a été inoculée sur chacune des plantes pré-germées. La bactérie a été cultivée sous agitation (180 rpm), sur la nuit, à 28°C, dans le milieu Nfbm contenant de la gentamycine (25 µg ml^{-1}). Après centrifugation, le culot a été resuspendu dans le volume de NaCl 0,8 % nécessaire pour obtenir une suspension contenant 10^7 cellules ml^{-1}. Pour chaque plante, deux plantules stériles (2 j de

germination) ont été déposées par boîte d'eau gélosée (8 g l^{-1} de Plant Agar) et deux boites ont été inoculées pour chaque condition (boîte 120x120x17 pour le riz et le blé, boîte 245x245x25 pour le maïs). 100µl de la suspension ont été déposés sur les plantules stériles (après 2 j de germination). Le même volume de NaCl 0,8 % stérile a été déposé pour les témoins non-inoculés. Les systèmes ont été incubés dans une chambre climatique comme décrit précédemment.

Les observations microscopiques ont été réalisées sur des échantillons (1 à 2 cm) de la zone apicale, de la zone pilifère et de la zone d'émergence des racines secondaires, comme décrit par Pothier *et al.* 2007. Elles ont été réalisées à l'aide d'un microscope 510 Meta (Carl Zeiss, Le Pecq, France) équipé de lasers argon-crypton et He-Ne (excitation à 488 nm et observation à 510-531 nm). Les images obtenues en fluorescence ont été superposées aux images de racines en lumière transmise à l'aide du logiciel LSM Image Browser Version 4.2 (Carl Zeiss).

Chapitre III :

Expression des gènes du riz au cours de la coopération avec *Azospirillum*

Le profilage de l'expression des gènes du riz inoculé avec *Azospirillum* révèle une réponse transcriptomique souche-dépendante

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En préparation

Transcriptome profiling reveals a strain-dependent response of rice to *Azospirillum* inoculation

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Abstract

Inoculation of *Azospirillum*, a major phytostimulator of cereals, results in modification of root architecture and higher nutrient uptake by the plant, and ultimately in enhancement of crop yield. *Azospirillum* also increases root exudation, modifies the chemical structure of root cell wall, alters secondary metabolism (in rice and maize), and can trigger plant resistance. Plant growth-promoting effects of *Azospirillum*, mainly attributed to phytohormones biosynthesis, depend both on host plant genotypes and bacterial strains, pointing towards a certain degree of specificity. Many studies have assessed the impact of PGPR on plant morphological traits but little is known about changes induced at the molecular level. Hence, this study aims at characterizing genetic determinants regulated in rice roots at an early stage of interaction with *Azospirillum,* considering possible favored interaction between a strain and its original host cultivar. Genome-wide analyses of roots gene expression of *Oryza sativa japonica* cultivar Cigalon and Nipponbare, were performed by using microarrays seven days post inoculation with *A. lipoferum* 4B (isolated from Cigalon roots) or *Azospirillum sp.* B510 (isolated from Nipponbare) and compared to the respective non-inoculated condition. By selecting a threshold foldchange value of 2, a total of 7384 genes appear to be regulated in rice roots, which represent about 16 % of total rice genes. Among these, only 49 genes are regulated by both *Azospirillum* strains in both rice cultivars. Each strain/cultivar combination displays specific expression profiles highlighting a strain-specific response of rice. The most important changes are observed with B510, a strain displaying endophytic properties. Fewer genes implicated in response to stress and plant defense are regulated in host combinations (Cig_4B and Nip_B510) than in non-host combinations (Cig_B510 and Nip_4B). Results also suggest that strain-dependent effects on 1-aminocyclopropane-1 carboxylate (a precursor of ethylene) pathway and ethylene signaling occur during the *Azospirillum*-rice cooperation.

Keywords: *Azospirillum*, Cigalon, Nipponbare, plant growth-promoting rhizobacteria, rice, transcriptomics.

III.1. Introduction

Rhizodeposition supports growth of a wide range of microorganisms able to establish intimate interactions with plant roots. In the case of parasitism, nutritional requirements of the microbe partner are supported at the expense of plant development and reproduction (O'Brien *et al.* 2011; Schumacher and Tundzynski 2012). In the case of mutualism, the interaction leads to a nutritional exchange so that costs and benefits are reciprocally shared by both microbial and plant partners (Odum 1971; Smith and Read 2008). Whether engaged in a parasitic or mutualistic interaction, the microbial partner is perceived as an intruder and the success of the adaptation strategy depends on the microbe's ability to bypass defense mechanisms and invade plant tissues (Soto *et al.* 2009). Cooperation involving Plant Growth-Promoting Rhizobacteria (PGPR) results in improvements of plant growth and health; however, the invasion of root tissues is not a critical step in successful interaction as several efficient strains are described as root-surface colonizers (Lugtenberg and Kamilova 2009; Chamam *et al.* 2013). If mechanisms directly implicated in plant growth-promotion have been extensively studied, most of these works have assessed the impact of PGPR on plant morphological traits and little is known about changes induced at the molecular level (Bashan and de Bashan 2010; Contesto *et al.* 2010; Galland *et al.* 2012; van de Mortel *et al* . 2012; **Annexe I**).

For more than fifty years, PGPR of a wide range of genera including *Acetobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Herbaspirillum*, *Phyllobacterium* or *Pseudomonas* have been known for stimulating the growth of numerous host plants (Desbrosses *et al.* 2009; Kumar *et al.* 2011; Lugtenberg and Kamilova 2009; Richardson *et al.* 2009; Saharan and Nehra 2011). In particular, the genus *Azospirillum* constitutes an important phytostimulator and an increasing number of field trials are undertaken, principally in India and Latin America where several *Azospirillum* inoculants are now commercialized (Steenhoudt and Vanderleyden 2000; Bashan *et al.* 2004; Fuentes-Ramirez and Caballero-Mellado 2005). In most cases successful inoculation results in roots and shoots morphological changes, yield enhancements and plant nutrition improvements (Richardson *et al.*, 2009; **Annexe I**). If the phytostimulating effect of *Azospirillum* was originally attributed to its

ability to fix atmospheric nitrogen, it is now admitted that the modulation of the phytohormonal balance is the most important mechanism resulting in the modification of root architecture and higher nutrient uptake by the plant (Baldani *et al.* 1979; Prigent-Combaret *et al.* 2008; Steenhoudt and Vanderleyden 2000, Somers *et al.* 2004). Besides morphological changes, *Azospirillum* also increases root exudation and modifies the chemical structure of root cell wall (Heulin *et al.* 1987, El Zemrany *et al.* 2007). In addition, *Azospirillum* was found capable of increasing the resistance of the host plant against pathogen through mechanisms independent of salicylic acid signaling (Yasuda *et al.* 2009). Investigation on maize secondary metabolism revealed that major qualitative and quantitative modifications occur following *Azospirillum* inoculation (Walker *et al.* 2011). Moreover, these modifications depend on bacterial strain/maize cultivar combinations suggesting that a genotype specific perception of *Azospirillum* occurs during the cooperation with maize. These observations were recently strengthened by a study made on two rice cultivars, Cigalon and Nipponbare, after the inoculation of two *Azospirillum* strains isolated from each cultivar (Chamam *et al.* 2013): *A. lipoferum* 4B isolated from Cigalon roots (Thomas-Bauzon *et al.* 1982) and *Azospirillum sp.* B510 isolated from Nipponbare (Elbeltagy *et al.* 2001). Profiling of secondary metabolites and morphological measurements evidenced that the impact of *Azospirillum* differ according to strain/cultivar combinations and that a specific interaction leading to a stronger phytostimulation occur between a strain and its original host cultivar. In addition, the endophyte strain B510 was shown to trigger a systemic response, as metabolic changes were observed in both roots and shoots. However, whether or not, perception of *Azospirillum* involved plant immune response remains an unanswered question and regulatory mechanisms underlying host-specific metabolic changes remain to be unraveled.

In this context, our study aims at characterizing genetic determinants regulated in rice roots at early stage of the interaction with *Azospirillum,* considering possible favored interaction between a strain and its original host cultivar. Thus, genome-wide analyses of root gene expression of *Oryza sativa japonica* cultivar Cigalon and Nipponbare, were performed seven days post inoculation with *A. lipoferum* 4B or *Azospirillum sp.* B510 and compared to the respective non-

inoculated condition. A focus was made on genes potentially involved in plant defense, hormone signaling and plant development.

III.2. Results

Transcriptomic profiles were obtained using microarray and the following combinations were analyzed (3 independent replicates per combination): Cigalon/*A. lipoferum* 4B (Cig_4B) and Cigalon/*Azospirillum sp.* B510 (Cig_B510) compared to non-inoculated Cigalon; Nipponbare/*A. lipoferum* 4B (Nip_4B) and Nipponbare/*Azospirillum sp.* B510 (Nip_B510) compared to non-inoculated Nipponbare. Genes significantly regulated were selected using a *P*-value threshold of 0.05 and a fold change cutoff of 2 ($|Log_2(FC)|\geq 1$). According to the cultivar of which each strain was originally isolated, Cig_4B and Nip_B510 combinations constitute the interaction between a strain and its original host cultivar (that will be designed as host combinations), while Cig_B510 and Nip_4B interactions constitute interactions with non-host cultivars (that will be designed as non-host interactions).

III.2.1. Genetic proximity between both rice cultivars

The microarray was designed on the basis of the genome sequence of cultivar Nipponbare. To ensure that it could be used to hybridize cDNA obtained for both Cigalon and Nipponbare, the genetic proximity between both cultivars was analyzed by sequencing 8 genes (Os01g0615100, Os01g0915900, Os03g0103200, Os090471300, Os09g0471400, Os09g0471500, Os09g0255400 and the gene encoding actin) after PCR amplification from Cigalon DNA (at least 500 pb per gene). For all the sequenced genes, an identity of 100% was observed with the corresponding genes of Nipponbare. In addition, when comparing the non-inoculated Cigalon transcriptomic profile to the non-inoculated Nipponbare profile, only 193 genes appear to be differentially transcribed between the two cultivars (87 up-regulated and 106 down-regulated). This represents only 0.43 % of the targeted genes (i.e. 45,000 genes). Thus, the microarray was considered suitable for analysis and comparison of both Nipponbare and Cigalon transcriptomic profiles.

III.2.2. General features of rice-roots transcriptome profiling in response to *Azospirillum* inoculation

When considering the four combinations, a total of 7384 genes are regulated in rice roots, which represent about 16 % of total rice genes. Each strain/cultivar combination displays characteristic expression profiles highlighting a strain-specific response of the host plant. The most important changes are observed when strain B510, isolated from Nipponbare, is inoculated on Cigalon roots (Cig_B510), with 3865 regulated genes, equally induced and repressed (1993 up-regulated; 1872 down-regulated). Inversely, the inoculation of strain 4B on roots of its original cultivar Cigalon (Cig_4B) is followed by the regulation of only 1243 genes, mostly induced (1196 up-regulated; 43 down-regulated). When considering Nipponbare roots, the number of regulated genes is similar for Nip_4B and Nip_B510 combinations with respectively 2141 and 2539 regulated genes. However, these genes are mostly induced in Nip_4B (1965 up-regulated; 176 down-regulated) while they are repressed in Nip_B510 combination (203up-regulated; 2336 down-regulated).

Functional classification available for 20 % of the regulated genes evidences that *Azospirillum* inoculation induces important expression changes of genes implicated in i) primary metabolism, ii) transport, iii) regulation of transcription and iv) protein fate, a feature observed for the four combinations (**Figure 22**). However, for each category, the number of induced genes appears to be lower when considering host combinations. In addition, genes potentially involved in signal transduction and translation process seem quasi exclusively repressed in Nip_B510 while they are induced in Nip_4B. Fewer genes implicated in response to stress and plant defense are regulated in Cig_4B and Nip_B510 host combinations than in Cig_B510 and Nip_4B non-host combinations. Interestingly, strain B510 could induce the repression of a more important number of these latter genes than strain 4B, particularly on its original host cultivar Nipponbare.

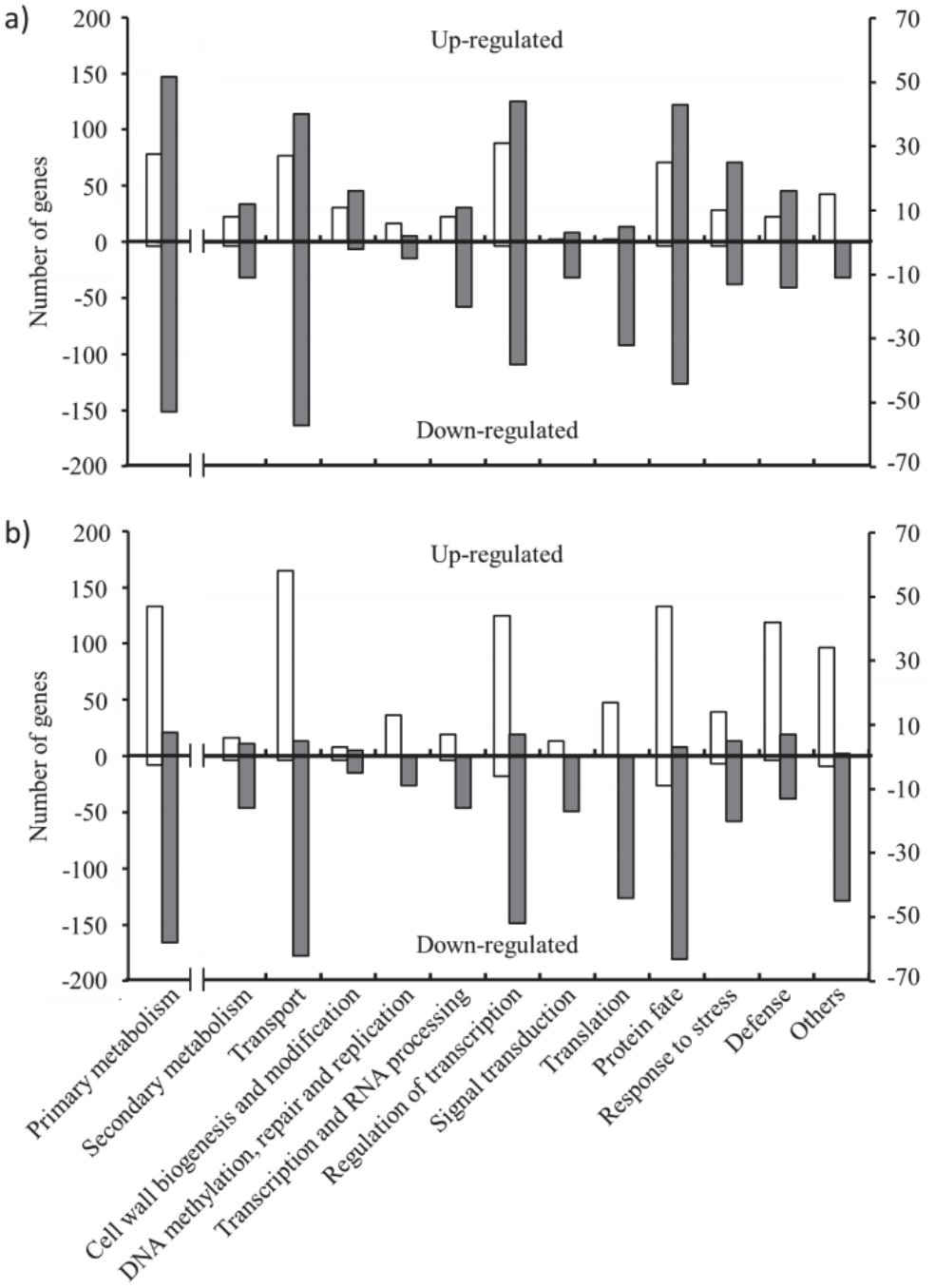

Figure 22 : Functional classification of the significantly regulated genes in *Oryza sativa* L. *japonica* in response to *Azospirillum* inoculation.

a) Genes significantly regulated in cultivar Cigalon, b) Genes significantly regulated in cultivar Nipponbare. White bars represent genes regulated by *A. lipoferum* 4B, grey bars represent genes regulated by *Azospirillum sp.* B510. Genes were classified according to Biological Process assignation taken from the Rap-DB database. Genes with no Biological Process assignation represent about 80% of regulated genes for each condition. Category "Others" includes principally genes implicated in photosynthesis, exocytosis, sexual reproduction, cytoskeleton organization, cell cycle and cell death.

Comparison of expression profiles obtained for the four combinations unveils 15 categories of regulated genes discriminated regardless of the expression levels (induced or repressed) (**Figure 23**). Four of these categories comprise genes that are regulated in only one of the four combinations and will be considered as combination-specific genes. This represent 329 genes for Cig_4B, 2025 genes for Cig_B510, 1497 genes for Nip_B510 and 1448 genes for Nip_4B. Two other categories regroup genes that are regulated by both strains in only one of the two cultivars and represent cultivar-specific genes (665 genes for Cigalon and 158 genes for Nipponbare). Inversely, two categories comprise genes that are regulated by only one strain in both cultivars, considered as strain-specific genes (651 genes for *Azospirillum sp.* B510 and 55 genes for *A. lipoferum* 4B). Two additional categories include genes regulated in both host combinations Cig_4B and Nip_B510 (22 genes) or in both non-host combinations Cig_B510 and Nip_4B (264 genes). One category contains 49 genes regulated by both *Azospirillum* strains in both rice cultivars. Finally, the four remaining categories comprise genes that are regulated in three of the four combinations and will no longer be discussed in the current analysis.

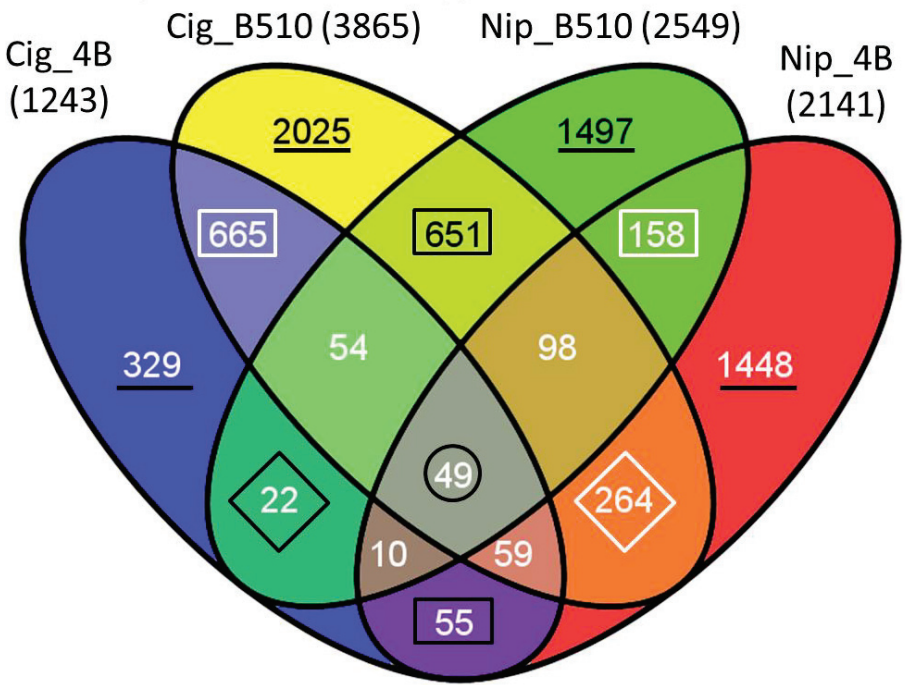

Figure 23 : Venn diagram representing differentially expressed genes (P_{adj}<0.05 and |Log$_2$ (FC)|≥1).
Circled number represents genes regulated by both *Azospirillum* strains in both rice cultivars. Numbers in black rectangles represent strain-specific genes. Numbers in white rectangles represent cultivar-specific genes. Numbers in black diamond represents genes regulated in both host combinations. Numbers in white diamond represents genes regulated in both non-host combinations. Underlined number represents combination-specific genes.

The microarray data were confirmed by analyzing expression levels of 14 representative genes using reverse transcription quantitative polymerase chain reaction (RT-qPCR) (**Table VII**). This includes seven up-regulated genes and seven down-regulated genes belonging to seven of the 15 categories described in **Figure 23**. These results show that the array data agree well with the RT-qPCR data.

Table VII. Validation of microarray data by RT-qPCR.

Gene		FC Cig_4B		FC Cig_B510		FC Nip_4B		FC Nip_B510	
		Log_2 (qPCR)	Log_2 (array)	Log_2 (qPCR)	Log_2 (array)	Log_2 (qPCR)	Log_2 (array)	Log_2 (qPCR)	Log_2 (array)
Significantly regulated in all condition									
Os09g0358000	Tyrosine kinase domain	2.27	1.77	2.53	2.46	1.87	3.01	2.06	1.32
Significantly regulated in Cigalon_4B and NP_4B only									
Os09g0417800	WRKY transcription factor 62	1.49	1.75	0.66	–	1	1.88	-0.01	–
Os11g0686900	Similar to NB-ARC domain	0.83	1.16	-0.23	–	1.74	2.4	nd	–
Significantly regulated in Cigalon_B510 and NP_B510 only									
Os06g0115600	Common symbiosis signaling (SYM) pathway	-0.09	–	-2	-1.84	0.01	–	-1.18	-1.47
Os05g0583000	Similar to WRKY8	0.32	–	2.92	1.71	0.16	–	2.24	1.39
Os12g0139400	A-type response regulator, cytokinin signaling	-0.15	–	-2.84	-1.78	-0.09	–	-2.94	-1.1
Os11g0143300	A-type response regulator, cytokinin signaling	0.15	–	-1.09	-1.95	0.04	–	-2.25	-1.62
Os02g0805100	Similar to auxin-responsive protein IAA12	-0.01	–	-1.15	-1.34	-0.23	–	-2.47	-1.16
Significantly regulated in Cigalon_4B and Cigalon_B510 only									
Os05g0196600	Similar to ACC synthase	0.38	1.33	0.95	1.63	-0.15	–	0.16	–
Significantly regulated in Cigalon_4B only									
Os08g0136100	Homeobox-leucine zipper domain	0.87	1.11	0.25	–	0.28	–	-0.14	–
Significantly regulated in Cigalon_B510 only									
Os06g0179200	Similar to Nodulin-like protein	0.16	–	-1.6	-1.78	0.18	–	0.12	–
Os08g0499300	WRKY transcription factor 30	0.1	–	0.52	1.34	0.12	–	-0.06	–
Significantly regulated in NP_B510 only									
Os01g0904700	B-type response regulator, cytokinin signaling	-0.12	–	-0.07	–	-0.04	–	-1.09	-1.21
Os05g0515400	Similar to auxin response factor 14	-0.09	–	0.1	–	-0.09	–	-1.15	-1.42
Reference gene									
Os03g0718100	Actin								

III.2.3. Genes regulated by both *Azospirillum* strains in both rice cultivars

A total of 49 regulated genes (including 22 genes of unknown function) are common to the four combinations (**Figure 23, Table VIII**). 34 of these genes (12 genes of unknown function) are induced in the four combinations and could be defined as plant markers of *Azospirillum*-rice interactions. Thus, it appears that a Pathogenesis-Related gene orthologue to PR10 of *Brachypodium* is induced in the four conditions (Os12g0555200). Similarly, three genes containing Serine/Threonine kinase domain (Os07g0537900, Os08g0501500, Os08g0203400) and one gene containing Tyrosine kinase domain (Os09g0358000) are induced by both strains in both cultivars, highlighting the involvement of protein kinases in signal transduction of inoculated roots. Common signal transduction could also involve phytohormones signaling as revealed by the induction of a terpene synthase implicated in gibberellin synthesis (Os04g0178300), and other signals such as phytoalexin (Os11g0474800). Two genes encoding Cys-Rich domain containing protein (Os02g0579800, Os02g0580000) and an orthologous gene to AT1G59950 of *Arabidopsis thaliana* (Os03g0237100), which are potentially implicated in stress response, as well as a transcription factor (Os02g0580000) are also induced in the four combinations.

Ten genes (including six encoding proteins of unknown functions) are induced in both combinations involving Cigalon but repressed in both combinations involving Nipponbare. Two genes are potentially involved in transcriptional regulation (Os01g0911700, Os10g0429200). This includes a transcription factor containing a B3 domain, potentially involved in plant development (Os01g0911700). These results highlight a combination-specific response of rice roots inoculated with *Azospirillum*.

Table VIII. List of the 49 genes significantly regulated for both strains in both rice cultivars.

Gene	$Log_2(FC)$*#				GeneDescription	Orthologous gene in *Arabidopsis thaliana*¥
	Cig_4B	Nip_4B	Cig_B510	Nip_B510		
Os01g0952800	1.70	3.32	2.75	2.65	Transcription factor with Achaete-scute related domain	AT3G56970
Os10g0429200	1.46	-1.39	2.13	-1.79	BTB/POZ fold domain containing protein	AT3G06190
Os07g0669600	1.69	1.48	2.02	-1.19	Conserved hypothetical protein	AT5G18540
Os12g0422800	2.04	-1.64	2.17	-1.03	Conserved hypothetical protein	_
Os11g0671600	1.58	-1.88	2.04	-1.62	Conserved hypothetical protein	AT5G28780
Os10g0195250	1.24	2.08	2.01	1.68	Conserved hypothetical protein	_
Os08g0263600	1.32	-2.22	1.59	-2.02	Conserved hypothetical protein	_
Os11g0262600	1.31	2.45	2.29	2.29	Conserved hypothetical protein	_
Os01g0608101	1.33	2.73	2.42	2.39	Conserved hypothetical protein	
Os06g0293500	2.40	3.26	2.62	1.73	Conserved hypothetical protein	_
Os02g0582900	2.20	4.95	3.77	4.98	Conserved hypothetical protein	_
Os02g0817900	-1.04	1.15	-1.89	-1.75	Cytochrome P450 family protein	AT1G31800
Os02g0569400	1.54	2.42	2.23	1.78	Cytochrome P450 family protein	AT2G45570
Os08g0501500	1.03	1.27	2.26	1.70	OsWAK receptor-like protein kinase	AT1G21240
Os04g0268300	1.33	-1.36	1.04	-1.03	Conserved hypothetical protein	_
Os12g0225300	-1.27	1.37	-1.96	-1.18	Conserved hypothetical protein	AT4G38180
Os12g0265701	-1.28	1.46	-1.23	-1.27	Conserved hypothetical protein	
Os08g0547500	1.21	-1.47	1.36	-1.35	Kinesin motor domain containing protein	AT3G51150.1
Os06g0718400	1.39	1.68	1.66	1.53	Plastocyanin-like domain containing protein	AT5G26330.1
Os12g0236100	1.17	2.03	1.70	1.60	Conserved hypothetical protein	AT3G01810
Os09g0358100	1.27	2.05	1.79	1.05	Senescence-induced receptor-like serine/threonine kinase	AT1G51790.1
Os01g0811001	1.00	2.14	-1.56	-1.37	Conserved hypothetical protein	AT1G51870.1
Os02g0594232	1.18	2.28	2.09	1.80	Conserved hypothetical protein	
Os01g0495701	1.14	2.60	2.02	2.12	Conserved hypothetical protein	
Os01g0647200	1.07	2.97	1.91	1.71	Conserved hypothetical protein	_
Os02g0791300	1.41	3.00	2.91	2.58	Conserved hypothetical protein	_

Os12g0171801	1.12	2.77	2.75	1.64	Hypothetical protein	
Os03g0103033	1.54	-1.65	2.04	-2.25	Hypothetical protein	
Os03g0307300	1.34	3.33	2.29	2.54	Nicotianamine synthase 1 (EC 2.5.1.43)	AT1G09240
Os07g0258400	1.61	2.25	2.60	1.70	Putative metal transporter Nramp6	AT1G80830
Os08g0203400	1.66	4.69	2.54	3.59	Protein kinase/ core domain containing protein	AT1G56130
Os07g0190000	1.69	3.02	2.77	2.85	Putative 1-deoxy-D-xylulose 5-phosphate synthase	AT4G15560
Os01g0191200	1.34	3.28	2.18	3.09	Putative HAD phosphatase	AT4G29260
Os02g0580000	1.51	2.80	1.78	1.32	Cys-rich domain containing protein	AT5G35525
Os04g0178400	1.92	3.56	2.60	2.82	Putative Cytochrome P450	AT3G26310
Os06g0486800	1.10	2.31	1.84	1.42	Putative mitochondrial formate dehydrogenase (EC 1.2.1.2)	AT5G14780
Os02g0579800	1.49	3.08	1.75	1.91	Cys-rich domain containing protein	AT1G14870
Os11g0474800	2.83	2.81	3.20	2.57	Putative stemar-13-ene synthase	AT1G79460
Os04g0178300	1.25	3.22	2.39	2.91	Putative syn-copalyl diphosphate synthase	AT4G02780
Os03g0237100	1.42	3.43	2.40	2.94	Putative NADPH-dependent codeinone reductase (EC 1.1.1.247)	AT1G59950
Os07g0416900	2.76	3.78	2.35	2.43	Omega-6 fatty acid desaturase	AT3G12120
Os09g0358000	1.77	3.07	2.71	2.51	Tyrosin kinase domain containing protein	AT1G51810
Os04g0155200	1.01	-1.75	1.82	-1.49	Putative uncharacterized protein, similar to OSIGBa0093M15.1 protein	_
Os07g0664000	1.52	2.24	2.59	1.85	Putative short chain dehydrogenase/reductase	AT3G26760
Os01g0212100	1.50	-1.51	1.47	-1.33	Putative ATP-dependent RNA helicase	AT4G18465
Os12g0555200	2.22	3.97	2.77	3.31	Putative pathogenesis-related Bet v family protein	_
Os03g0129800	1.01	1.79	1.01	1.58	Uncharacterized protein, similar to InsB from *Escherichia coli*	AT1G14590
Os07g0537900	1.12	1.24	1.83	1.62	Ser/Thr kinase receptor domain	_
Os01g0911700	1.07	-1.36	1.26	-1.30	Transcription activator VP1-rice	AT3G24650

*Fold-change values are indicated in red for genes that are up-regulated in the four combinations.
#Fold-change values are indicated in green for genes that are induced in Cigalon but repressed in Nipponbare.
¥ Gene labels are indicated in black when an orthologous is described in MSU Rice Genome Annotation Project Database (rice.plantbiology.msu.edu/index.shtml) and in blue when a similar sequence was obtained with the Blast tool in The Arabidopsis Information Resource (http://www.arabidopsis.org/index.jsp).

III.2.4. Strain-specific genes, regulated only by one *Azospirillum* strain in both rice cultivars

As illustrated in **Figure 23**, a total of 55 genes are regulated only by strain 4B in both rice cultivars and 651 genes are regulated only by strain B510 in both rice cultivars (**Additional File 1, Annexe V**). These strain-specific genes are of particular interest as they may be related to lifestyle differences between the two strains. Indeed, strain 4B was shown to colonize only root surface while strain B510 is able to colonize the outer layers of root-tissues (Chamam *et al.* 2013).

Genes specifically regulated by the surface-colonizing strain 4B are all induced in both cultivars, except the gene Os10g0332000 that is induced in Cig_4B and repressed in Nip_4B. This gene encodes the arbuscular mycorrhizal specific marker 34 (AM34) containing an UDP-glycosyl transferase domain (PF00201). Among the 54 genes induced in Cig_4B and Nip_4B, two genes encode proteins similar to peroxidases (Os03g0368000, Os03g0368300) potentially involved in oxidative stress response. The induction of two WRKY transcription factors (Os03g0335200, Os09g0417800) and two protein kinases (Os09g0471550, Os11g0667000) suggests that strain-specific transcriptional regulation occurs in rice roots in response to *Azospirillum* inoculation.

A larger number of genes, divided into three sets, are specifically regulated by the endophyte strain B510. The first set comprises 554 genes that are repressed in both Cig_B510 and Nip_B510 combinations. A substantial proportion of these genes are involved in sugar, lipid and energy metabolism. While a gene encoding a thioredoxin domain is specifically induced by strain 4B in both cultivars, strain B510 seems to repress four thioredoxin related genes (Os02g0567100, Os07g0657900, Os10g0472400 and Os12g0188566). The repression of a putative indole synthase (Os03g0797000), two putative auxin responsive proteins (Os02g0805100, Os11g0523800) as well as two response regulators involved in cytokinin signaling (Os11g0143300, Os12g0139400) and an isochorismate synthase (Os09g0361500), required for salicylic acid synthesis, suggest that a B510-specific hormone signaling occur in rice roots. In addition, some genes potentially related to cell division and plant development among which two germin-like protein precursors

(Os08g0188900, Os08g0189100) and a caleosin related gene (Os06g0254300) are down-regulated. The strain-specific adjustment of regulatory functions afore noticed for strain 4B is likewise observed for strain B510, as revealed by the repression of 4 putative transcription factors, 3 genes encoding serine/threonine kinases and two genes encoding protein containing Zinc finger C2H2-like domain (Os12g0581900) and TAZ-type domain (Os01g0893400) respectively. Finally, three genes containing a NB-ARC domain (Os01g0269800, Os12g0199100 and Os12g0468300), generally associated to Resistance genes (R genes), a putative Pathogen-Related protein (Os12g0555100) and a gene involved in common symbiosis signaling (SYM) pathway (Os06g0115600) are repressed in both Cig_B510 and Nip_B510 combinations.

The second set includes 38 genes that are induced in both Cig_B510 and Nip_B510. This comprises genes potentially implicated in ethylene signaling (Os03g0860100), and jasmonate signaling (Os04g0395800, Os06g0313320), again suggesting the potential involvement of a strain-specific hormone response in rice roots. While two genes encoding germin-like proteins are specifically repressed (see above), two other genes encoding germin-like proteins (Os04g0617900, Os08g0190100) are specifically induced. Among the remaining genes, a gene encoding a transcriptional regulator similar to WRKY8 (Os05g0583000) is also induced in both Cig_B510 and Nip_ B510.

Finally, the third set comprises 59 genes of particular interest as they display opposite regulation, i.e. induced in Cig_B510 but repressed in Nip_B510. Three of these genes encode proteins involved in transport including a MDR-like ABC transporter (Os08g0153000), a sucrose transporter (Os03g0170900) and a putative proton-dependent oligopeptide transporter (Os11g0282800). None of the genes belonging to this category are known to be involved in plant defense or plant-microbe interactions. Interestingly, no gene displays the opposite pattern of regulation, i.e. repressed in Cig_B510 and induced in Nip_ B510.

III.2.5. Cultivar-specific genes, regulated by both *Azospirillum* strains in only one rice cultivars

Besides genes that display a strain-specific expression profile, 665 genes are regulated by both strains only in cultivar Cigalon and 158 genes are regulated by both strains only in cultivar Nipponbare (**Figure 23; Additional file 2, Annexe V**). These cultivar-specific genes represent genes that could be related to rice genotype-specific perception of *Azospirillum* strains. For both cultivars, three patterns of expression profiles can be distinguished: (i) genes induced in response to both *Azospirillum* strains, (ii) genes repressed in response to both *Azospirillum* strains, and (iii) genes displaying an opposite pattern of expression; but they are all induced in response to strain 4B and repressed in response to strain B510 (**Table IX**).

Table IX. Number of genes regulated for each regulation pattern observed for cultivar-specific genes.

	Expression pattern	Number of regulated genes	
		Cigalon	Nipponbare
i)	induced in response to both *Azospirillum* strains	639	48
ii)	repressed in response to both *Azospirillum* strains	23	33
iii)	opposite pattern of expression *	3	77
	Total	665	158

* induced in response to 4B and repressed in response to B510

At least eleven genes potentially associated to plant development are induced in both Cig_4B and Cig_B510: 3 transcription factors containing GRAS domain (Os05g0500600, Os11g0139600, Os12g0162700) that could be involved in plant development; 3 genes potentially associated to embryogenesis (Os04g0510900, Os09g0482550, Os04g0588200); an orthologous gene to AT2G39700 of *A. thaliana* (Os03g0822000) that belongs to the alpha-expansin family; a gene encoding a xyloglucan fucosyltransferase implicated in cell-wall biogenesis (Os08g0334900); a

gene encoding a protein containing a "no apical meristem" (NAM) domain associated to plant development proteins (Os01g0667000); and finally a gene encoding a protein similar to the clock-controlled FKF1 of *A. thaliana* (AT1G68050). As for cultivar Nipponbare, only one gene, orthologous to AT2G40610 of *A. thaliana* (Os01g0823100) belonging to the alpha-expansin family, is induced by both strains. All the cultivar-specific genes potentially involved in plant defense response or plant-microbe interactions are induced in response to both *Azospirillum* strains. This represents two genes encoding NB-ARC domain containing proteins for cultivar Nipponbare (Os01g0721400, Os02g0282000) and five other related genes for cultivar Cigalon (Os03g0254000, Os05g0216950, Os08g0235651, Os11g0223400, Os11g0605600). In addition, three genes associated to disease resistance (Os01g0536501, Os08g0202400, and Os11g0179400), a gene similar to Pathogenesis-related genes (Os07g0244100) and three WRKY transcription factors (Os03g0758950, Os05g0478800, Os06g0649000) potentially involved in plant immunity are up-regulated in both Cig_4B and Cig_B510.

For cultivar Cigalon a gene encoding a cytokinin-O-glucosyltransferase (Os03g0745100), a gene encoding a protein similar to 1-aminocyclopropane-1-carboxylic acid synthase and an auxin-induced gene (Os04g0537100) are up-regulated in response to both strains. Interestingly, an auxin-responsive gene (Os11g0221000) displays an opposite regulation when considering Nip_4B and Nip_B510. These results suggest that a cultivar-specific phytohormone signaling could occur and highlight the complexity of phytohormone signaling in *Azospirillum*-rice interaction.

The repressions impact genes involved in metabolic processes, transport mechanisms and many genes of unknown functions but no genes potentially involved in plant defense response. For cultivar Nipponbare, one gene encoding an alpha-expansin potentially implicated in plant development and a gene similar to indole-3-acetate beta-glucosyltransferase are repressed by both *Azospirillum* strains.

III.2.6. Genes regulated only in host and non-host combinations

To understand whether adaptation strategies could be specific to either host or non-host combinations, we considered the 22 genes that are regulated only in both host interactions (Cig_4B and Nip_B510), and the 264 genes that are regulated only in both non-host interactions (Nip_4B and Cig_B510) (**Additional file 3, Annexe V**).

Among the 22 genes shared only by Cig_4B and Nip_B510, only one gene similar to a peroxidase (Os07g0638600) displays a similar regulation in both conditions. Indeed, while this gene is repressed in both Cig_4B and Nip_B510, the 21 remaining genes are induced in Cig_4B and repressed in Nip_B510. One of these 21 genes encodes an AWPM-19-like family protein (Os01g0247900) that could suggest an opposite regulation of abscisic acid levels in each of the host combinations.

Similarly, only 50 of the 264 genes shared only by Cig_4B and Nip_B510 display similar regulations in both conditions. Among the 214 remaining genes, we can distinguish 23 genes that are repressed in Nip_4B but induced in Cig_B510 and inversely 191 genes that are induced in Nip_4B but repressed in Cig_B510.Two of these genes, one encoding a F-box domain containing protein (Os05g0175800) and the other encoding a protein similar to tropinone reductase are down-regulated. The 48 remaining are up-regulated in both Cig_4B and Nip_B510. This comprises two genes implicated in successive steps of ethylene synthesis (Os06g0524900, Os09g0451400), a NB-ARC domain containing gene (Os06g0524900) and a gene encoding an expansin (Os05g0276500). Several genes implicated in oxido-reductive processes (Os01g0327000, Os07g0164900, Os12g0260500) as well as a gene encoding a glycoside hydrolase (Os09g0395600) and a gene encoding an UDP-glucuronosyl transferase (Os01g0597800) are also induced.

III.2.7. Combination-specific genes, regulated only by one strain in one cultivar

Combination-specific genes represent about 72 % of the 7384 regulated genes. The proportion of these genes that are down-regulated varies considerably between the combinations as they represent 10 of the 329 Cig_4B-specific genes

(3.0 %), 72 of the 1448 Nip_4B-specific genes (5.0 %), 1047 of the 2025 Cig_B510-specific genes (51 %), and 1471 of the 1497 Nip_B510-specific genes (98 %) (**Additional file 4, Annexe V**).

The majority of genes involved in disease resistance are induced: all of the four Cig_4B-specific genes and 33 of the 34 Nip_4B-specific. Conversely, in response to strain *Azospirillum* sp. B510, this type of genes is mainly down-regulated: 12 of the 21 Cig_B510-specific genes and eight of the nine Nip_B510-specific genes.

As for genes related to hormone signaling, two ethylene response factors and two genes involved respectively in auxin and cytokinin metabolism are down-regulated among Cig_B510-specific genes while three genes encoding respectively an ACC oxidase, a cytokinin glucosyltransferase and a gibberellin regulated protein are induced. For Nip_B510-specific, all the genes potentially involved in hormone signaling are repressed while they are all induced in both Cig_4B-specific and Nip_4B-specific. Similar combination-specific expression profiles are observed for transcription factors, including several WRKY domain containing genes and GRAS domain containing genes, as well as for genes related to embryogenesis and genes encoding expansin.

Interestingly, several genes involved in plant secondary metabolism display a combination-specific profile. Indeed, two genes encoding respectively a protein similar to cinnamyl alcohol dehydrogenase (Os11g0529036) and a protein similar to chalcone and stilbene synthases (Os04g0612700) are specifically induced in Cig_4B-specific. Similarly, two genes potentially involved respectively in geranylgeranyl-diphosphate (Os06g0656500) and coumaroyl-CoA (Os02g0668100) synthases are induced in Nip_4B-specific. On the contrary, genes implicated in phenyl propanoyl pathway (Os01g0528800, Os01g0828100) as well as genes related to terpene biosynthesis (Os01g0703400, Os02g0458100) are repressed in Cig_B510-specific. Finally, the Cig_B510-specific combination displays an intermediate stage with the induction of a terpene synthase family gene (Os03g0361500) and the repression of three genes involved in alkaloid (Os10g0114300), steroids (Os02g0701600) and terpene (Os12g0491800) biosynthesis, respectively.

III.3. Discussion

Understanding the molecular basis of host-specific adaptations in PGPR-plant cooperation may help improving biofertilization strategies in the context of sustainable agriculture. Based on previous studies detecting metabolic and morphological changes of PGPR-inoculated plants at early stages (Chamam *et al.* 2013; Cassán *et al.* 2009; Walker *et al.* 2010, 2012), we undertook to analyze transcriptomic response of rice roots seven day after inoculation. To our knowledge, our study constitutes the first investigation of wide transcriptomic response of rice roots to PGPR-inoculation, by considering two cultivars and two *Azospirillum* strains.

Comparison of expression profiles obtained for the four combinations evidences a fine-tuned transcriptomic response depending on both *Azospirillum* and rice genotypes. As revealed by the high percentage (72 %) of genes regulated in one combination and the high number of genes shared by two combinations that display opposite regulation, strain/cultivar combination appears to be the most important constraint modifying transcriptomic changes in rice roots. Only 34 markers of the *Azospirillum*-rice cooperation, induced in the four combinations, were identified and further investigations should be undertaken to identify the effect of a larger range of *Azsopirillum* strains on the regulation of these markers. Besides combination-specific traits, expression profiles showed strain-specific and cultivar-specific characteristics, highlighting potential differences in the strategies of interaction. Indeed, whatever the cultivar, strain 4B causes few repressions while at least half of the genes regulated in response to strain B510 are repressed. In addition, only 54 genes display similar regulation in both combinations involving strain 4B whereas it represents 592 genes for those involving strain B510. Accordingly, strain-specific responses were observed when considering the respective impact of 4B and B510 on development and secondary metabolism of rice cultivars Cigalon and Nipponbare (Chamam *et al.* 2013). While 4B promotes shoot and root growth of both cultivars, B510 promotes development of cultivar Nipponbare exclusively. However, B510 was shown to be the only strain inducing a systemic response, as revealed by variation of secondary metabolite profiles of both shoots and roots. These strain-

specific responses could be due to differences in strain lifestyle as 4B colonize only the surface of rice roots while B510 has the ability to colonize the cortex layers in rice (Chamam *et al.* 2013; Elbeltagy *et al.* 2001; Thomas-Bauzon *et al.* 1982). Besides their impact on plant growth, endophytic PGPR induce stress and defense responses and the inoculation of B510 was shown to enhance resistance against rice blast disease and rice blight disease (Miché *et al.* 2006; Rosenblueth and Martinez-Romero2006; Yasuda *et al.* 2009). However, whether these changes were the result of major gene induction or repression was not addressed. Recently, a study on differential gene expression of rice roots evidenced decrease in expression of defense related protein PBZ1 and thionins in roots inoculated with the endophytic *Herbaspirillum seropedicae* (Brusamarello-Santos *et al.* 2011). Authors assumed that the bacteria modulate plant defense to allow the establishment of an efficient cooperation. Indeed, colonization of root tissues by bacteria depends on the balance between plant's ability to induce efficient defenses in response to the intrusive microbe and microorganisms' ability to bypass plant immunity (Pieterse *et al.* 2009).

We evidenced that several genes associated to plant defense mechanisms are still regulated seven days post inoculation. Particularly, a gene orthologue to PR10 gene of *Brachypodium* and a gene related to phytoalexin biosynthesis are induced whatever the strain/cultivar combination. PR genes and phytoalexins are known to be induced during PAMPs-triggered immunity (PTI), the first step of plant defense that involves Pattern Recognition Receptors (PRRs) (Chisolm *et al.* 2006; Pieterse *et al.* 2009). PRRs recognize universal microbial determinants such as flagellin, chitin, glycoproteins and lipoproteins designed as PAMPs/MAMPs for Pathogens/Microbes –Associated Molecular Patterns (Schwessinger and Zipfel 2008). Thus we hypothesized that *Azospiririllum* strains 4B and B510 may share a similar determinant able to induce a PTI-like response in both rice cultivars. Several NB-ARC domain containing genes are regulated on a strain/cultivar dependent manner, the most striking effect being observed for the non-host combination Nip_4B, with the induction of at least 20 NB-ARC genes. On the contrary, less than 5 NB-ARC genes are induced in the host combination Cig_4B. Moreover, it appears that most of NB-ARC genes regulated during interaction with strain B510 are down-regulated and that repression occurs for a higher number of NB-ARC genes in the Nip_B510

host interaction. NB-ARC domain is generally associated to R genes that are involved in Effector-Triggered Immunity (ETI) (Chisolm *et al.* 2006; Pieterse *et al.* 2009). Differences observed between host and non-host combination suggests that the way a strain is perceived by rice roots could have been subjected to long-lasting co-adaptation events between a strain and its original host cultivar. Such a hypothesis has already been proposed for *Azospirillum*-rice when considering changes in secondary metabolites composition of rice inoculated with strain 4B and B510, or transcriptomic response of strain 4B during the association with rice roots. The involvement of plant defense systems in PGPR-plant cooperations was mainly considered in the context of biocontrol agents and little is known about the perception of phytostimulating bacteria (Pieterse *et al.* 2009; Van Wees *et al.* 2008; Van Loon *et al.* 1998). Thus, the perception of biocontrol PGPR leads to the induction of long-lasting and broad-spectrum systemic resistance (Pieterse *et al.* 2009; Van Wees *et al.* 2008; Van Loon *et al.* 1998). Induced systemic resistance (ISR) is associated with priming effect for enhanced defense inducing a few reprogramming of plant transcriptome (Van Wees *et al.* 2008; Verhagen *et al.* 2004; Wang *et al.* 2005). In the case of phytostimulating PGPR, it was reported that members of genus *Azsopirillum* and *Burkholderia* induce defense response at a lower extent than pathogens (Bashan 1998; Bordiec *et al.* 2011). Moreover, induction of plant defense mechanisms was shown to control the establishment of compatible and incompatible interactions between plants and endophytic PGPR (Miché *et al.* 2006; Rosenblueth and Martínez-Romero 2006; Reinhold-Hurek et Hurek 2011). Thus differences between endophyte and surface colonizer effects on plant defense system should be further undertaken by studying the impact of other *Azospirillum* strains that display endophytic properties, such as *A. brasilense* Sp245. Moreover, plant immunity involved dynamic mechanisms and taking into account temporal changes in the expression of defense related genes remains an important issue to measure the sustainability of host combinations.

Modulating plant hormone balance is an important trait of phytostimulating PGPR (Richardson *et al.* 2009; Bashan and de Bashan 2010). Especially, several members of the genus *Azospirillum* are able to produce auxin, cytokinin and gibberellin involved in plant development (Richardson *et al.* 2009; Bashan and de

Bashan 2010). In the case of strains 4B and B510, genes related to indole-3-acetic acid (IAA) biosynthesis pathway, *ipdC*/*ppdC*, are absent from their genomes and 1-aminocyclopropane-1 carboxylate (ACC) deamination could be a relevant mechanism for hormone modulation and plant-growth promotion (Blaha *et al.* 2006; Prigent-Combaret *et al.* 2008). This property is encoded by *acdS* found in both pathogenic and non-pathogenic bacteria (Blaha *et al.* 2006). ACC is a precursor of ethylene, a gaseous hormone that represses root-growth and induces systemic resistance against pathogens (Bleecker and Kende 2000; Galland *et al.* 2012; Pieterse *et al.* 2009). It was proposed that bacterial deamination of ACC could lead to a decrease of ethylene levels in plant roots and consequently an increase in root development (Contesto *et al.* 2008; Galland *et al.* 2012; Glick 2005). In the current work, the most important regulations of genes related to ethylene biosynthesis and signaling are observed for B510, as illustrated in **Table X**.

Table X. Regulation of genes implicated in ACC biosynthesis and ethylene pathway.

Gene	Log_2 (FC)				Gene description
	Cig_4B	Nip_4B	Cig_B510	Nip_B510	
Os05g0196600	1.33		1.63		Similar to ACC synthase
Os02g0202000		1.32			Similar to ethylene responsive protein
Os07g0239400		1.42			Similar to ethylene-responsive protein
Os09g0451400		1.27	1.55		ACC oxidase
Os09g0453800		1.68	1.36		ACC synthase
Os09g0434500			-1.49		Similar to ethylene response factor
Os09g0287000			-1.47		Similar to ethylene-response factor
Os09g0451000			1.17		ACC oxidase
Os03g0860100			1.52	1.35	Similar to ethylene-response factor
Os11g0186900				-1.82	ACC oxidase
Os05g0155200				-1.41	Similar to ethylene receptor
Os06g0573900				-1.1	Similar to ACC oxidase

Four genes involved in ethylene biosynthesis through the ACC pathway (two ACC synthases and two ACC oxidases) are induced in Cig_B510 suggesting an increase in ethylene level in Cigalon roots. However, only three genes potentially involved in ethylene signaling are regulated, two being repressed and the other one being induced. In Nip_B510, two ACC oxidases are repressed and may lead to a decrease in ethylene. On the contrary, in Nip_4B an ACC synthase and an ACC

oxidase (both shared with Cig_B510 combination) and two ethylene responsive genes are induced. These results suggest that a strain dependent impact on ACC pathway and ethylene signaling occur in *Azospirillum*-rice cooperation.

Many plant hormones are involved in both plant growth and plant immunity, two physiological traits regulated by a network of interconnected signaling pathways (Pieterse *et al.* 2009). Among these, auxin was shown to contribute to both plant development and disease resistance in a pathway interconnected with salicylic acid signaling (Wang *et al.* 2007). Cross-communication between plant immunity and plant development may contribute to quick adaptation in a cost-efficient manner according to numerous trade-offs reported between growth rate and disease resistance (Pieterse *et al.* 2009; Walters and Heil 2007). Thus the strain-specific effect of *Azospirillum* on the regulation of auxin related genes must be taken into account to appraise the cost-benefit balance of each strain/cultivar cooperation. Strains 4B and B510 do not produce IAA; however, they are both able to produce a molecule that induces auxin-like response in *A. thaliana* (our unpublished results). While these two strains display similar characteristics for hormonal production, regulation of rice genes related to auxin signaling is strikingly different when considering each of the strain. Indeed, all these regulated genes except one are induced with strain 4B while they are mostly repressed with strain B510. Moreover several auxin-related genes display combination-specific expression profiles highlighting the complexity of hormone signaling networks involved in *Azospirillum*-rice cooperation. Thus, understanding how these networks are connected to both growth promotion and plant defense response appears to be an important issue to unravel mechanisms involved in beneficial interactions between PGPR and plants.

III.4. Material and Methods

III.4.1. Biological material

In this study, two rice (*Oryza sativa* L.) cultivars belonging to the Japonica group, cv. Cigalon (Centre Français du Riz, France) and cv. Nipponbare (J.B. Morel, BGPI, Montpellier, France) were inoculated with to diazotrophic strains of the genus *Azospirillum*: *A. lipoferum* 4B initially isolated from rice roots of the cv. Cigalon in

France (Thomas-Bauzon *et al.* 1982) and *Azospirillum* sp. B510 initially isolated from surface sterilized rice stems of the cv. Nipponbarre (Elbeltagy *et al.* 2001).

III.4.2. Seed sterilization, Plant inoculation and growth conditions

All these steps were performed as previously described (Drogue *et al.* submitted, **Chapitre II Partie 1**).

III.4.3. RNA extraction and cDNA synthesis

Three independent experiments were performed per condition. For each experiment, 30 plant root systems were pooled and frozen using liquid nitrogen. Root cell lysis was performed by grinding root systems with a mortar and pestle under liquid nitrogen. Total RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA samples were purified using RNeasy plant mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. RNA integrity was assessed using Agilent RNA 6000 Pico Kit (Agilent Technologies, Waldbronn, Germany) and the Agilent 2100 Bioanalyzer (Agilent Technologies) device.

In order to increase mRNA representation in RNA samples, total RNA were digested with mRNA ONLYTM Procaryotic mRNA isolation kit (Epicentre Biotechnologies, Madison, WI, USA) according to the provided protocol.

The microarray cDNA (3 independent samples per condition) was synthesized with the SuperScript® Double-Stranded cDNA Synthesis Kit (Invitrogen), using a mix (1:1) of random primers (Promega Corporation, Madison, WI, USA) and Oligo-dT(15) primers (Promega).

III.4.4. Microarray hybridization and data analysis

We designed an oligo microarray, which was produced by NimbleGenTM (Madison, WI, USA) derived of one which was described previously (Picault *et al.* 2009). This microarray is composed of about 385,000 60mers probes selected for their GC content, Tm and number of cycles needed to synthesize the oligo. This chip contains 90,000 probes representing 45,000 genes (2 probes per gene) of rice Oryza

sativa ssp. Japonica, based on the TIGR rice genome annotation version 3.1 genes (Yuan *et al.* 2005) and 290,000 probes representing all copies of LTR retrotranposons (Chaparro *et al.* 2007) and 1,000 bp of their flanking regions at the 3' and 5' side. The oligonucleotides have been designed at the 3' end of the genes to detect the readings of reverse transcriptase. On the other hand, the retrotransposons are represented throughout their length at the rate of a probe every 500 bp.

When it was possible, probes have been designed to be unique in the genome (i.e locus specific) to overcome the problems of oligonucleotides redundancy on the chip. When there were three mismatches during hybridization between a cDNA and an oligonucleotide, hybridization was considered stable enough to withstand the conditions of washing after the chip hybridization. The oligonucleotides are therefore regarded as locus specific when they are not matching elsewhere, but having at most three mismatches, which represents 5% of all oligonucleotides.

For each condition, three independent cDNA samples were labeled and hybridized by Roche Nimblegen according to their standard protocol. Data analysis was performed using Bioconductor microarray packages for R software (http://www.bioconductor.org/). The robust multi-array average (RMA) method associated with quantile normalization was applied (Irizarry *et al.* 2003; Bolstad *et al.* 2003). Analysis of variance with a false discovery rate adjustment method was realized (Benjamini and Hochberg 1995). The results of different treatment comparison were obtained in Log2-fold change. The oligonucleotides selected were those which present a two fold increase or decrease in expression, i.e, a log-fold change smaller or equal to -1 for down-regulation, and greater or equal to 1 for up-regulation. Oligonucleotides displaying P-value ≤0.05 for the statistical test were selected. For each cultivar, the respective uninoculated condition was used as control. All oligonucleotides differentially expressed were remapped to Os-Nipponbare-Reference-IRGSP version 1.0 (Rice Annotation Project, 2008) using BLAST.

III.4.5. RT-qPCR

For each condition, three independent RNA samples were used to validate gene expression level by performing reverse transcription quantitative real-time PCR (RT-qPCR). Validation was made on a group of 14 representative genes (**Table VII**) using LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) on a LightCycler® 480 Real-Time PCR System (Roche). The actin gene (Os03g0718100) showing an invariant expression was used as reference to normalize RT-qPCR values. After DNase I treatment, total RNA (800 ng) was used for cDNA synthesis using GoScriptTM Reverse transcription system (Promega) with oligo-dT(15) primer in accordance with the manufacturer's protocol.

DNA contamination was checked with reactions that lacked reverse transcriptase as negative controls. Specific primers were designed using Light Cycler Probe design Software 2.0 (Roche) with the following criteria: product size ranges 100-400 pb, primer size comprised between 17 and 22 bases, optimal primer Tm 60°C (**Table XI**). Real time PCR conditions were: a denaturation stage of 10 min at 95°C; an amplification stage of 45 cycles of 15 s at 94°C, 10 s at 60°C and 20 s at 72°C; and a melting curve stage of 5 s at 95°C and 1 min at 65°C increased to 97°C with a ramp rate of 0.11°C s^{-1}. All reactions were performed in 3 technical replicates and carried out in LightCycler 480 Multiwell plate 96 (Roche) with adhesive sealings foils (Roche) in a final volume of 10 µl containing 1 µl of each primer (5 µM), 5 µl of master mix and 3 µl of cDNA diluted 50 times. For each cultivar, the respective uninoculated condition was used as the calibrator condition and relative gene expression was calculated using the 2-ΔΔCt method (Livak and Schmittgen 2001).

Table XI. Specific primers for qPCR.

Gene	qPCR Primers
Os01g0904700	For: TGATAGCTGCGACGATCTTCA
	Rev: TCTGCATGGTCTTTGTCCAC
Os02g0805100	For: CGCTGGACCTGTGGTCA
	Rev: CTTCAGGCGTTTCACAGA
Os05g0196600	For: GGAGAGCCTATGATGAGGAC
	Rev: GGGTGCTCCCTCATGTATTC
Os05g0515400	For: CTAGCTTCAACTCTAGGGCAT
	Rev: GGAGCTGTCGCTTGGTTA
Os05g0583000	For: GGTGCGAATGACGATGAAC
	Rev: CAATAGCCGTGGTGACT
Os06g0115600	For: CAAGCAATGACTGTTTGTGAG
	Rev: GGATCCCATTCGAGTTAGG
Os06g0179200	For: CACACTTCATGTCTCTGTTGC
	Rev: CTGTTAAACAAGAGTGCGCC
Os08g0136100	For: GTTCCTGCGCTACTGCAA
	Rev: CCTTGGAGTAGCCGTTCG
Os08g0499300	For: CGCTTTCTCTGGGCTTTG
	Rev: TGTCGATGGCGTTGGTG
Os09g0358000	For: GCTGGATCAATGGGTTACA
	Rev: TCATAGTCATCCCCAAGTCG
Os09g0417800	For: GAGACCGTGAAGGATGGG
	Rev: TCGTAGGTCGCCACTAGC
Os11g0143300	For: TTCCATGTCCTGGCTGTG
	Rev: TCCCGGAATCAACAGTGGTA
Os11g0686900	For: CATGGCAGCATTCTTCCC
	Rev: ACTCGCTGTAATAGCCATTG
Os12g0139400	For: ATAGAGGCTCCGTTCCAT
	Rev: CCCGAATCAACAGTGGTTA
Reference gene (Actin)	
Os03g0718100	For: GGAGCGTGGTTACTCAT
	Rev: TGCCAGGGAACATAGTG

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Discussion Générale

Ces travaux de thèse constituent la première analyse de la réponse transcriptomique de chacun des partenaires de la coopération *Azospirillum*-riz. D'une part, nous nous sommes intéressés à l'effet de deux cultivars de riz, Cigalon et Nipponbare, sur l'expression des gènes d'*A. lipoferum* 4B sept jours après inoculation ainsi qu'à l'effet de cette souche sur l'expression des gènes de chacun des cultivars choisis comme modèles. D'autre part, afin de mettre en évidence les mécanismes potentiellement impliqués dans la spécificité de la coopération *Azospirillum*-céréales, l'effet du riz sur le transcriptome d'*A. lipoferum* 4B a été comparé à l'effet de deux autres céréales, le blé et le maïs. De même, nous avons étudié la spécificité du point de vue du partenaire végétal en considérant l'impact d'une seconde souche d'*Azospirillum* (*Azopirillum sp.* B510) sur l'expression des gènes des deux cultivars de riz.

Adaptation réciproque des partenaires de l'interaction *Azospirillum*-riz

Au-delà de l'effet d'*Azopirillum* sur la morphologie des plantes, de précédentes études ont montré son impact sur la composition des métabolites secondaires dans les feuilles et les racines du maïs et du riz (Walker *et al.* 2010 ; Chamam *et al.* 2013). Plus précisément, la souche *A. lipoferum* 4B induit des variations qualitatives et quantitatives de la composition des métabolites secondaires dans les racines du cultivar Cigalon à partir duquel elle a été isolée, alors qu'elles sont moindres dans le cas du cultivar Nipponbare (Chamam *et al.* 2013). Ces modifications sont également associées à une stimulation plus importante de la croissance du cultivar Cigalon. Cette même étude a montré que l'impact de la souche *Azsopirillum sp.* B510 sur les profils métaboliques et la croissance du riz étaient également plus importants sur son cultivar d'origine, le cultivar Nipponbare. Ces résultats ont conduit à l'hypothèse de la mise en place d'une interaction préférentielle entre une souche et son cultivar d'origine (Chamam *et al.* 2013).

Qu'il s'agisse du partenaire bactérien (*A. lipoferum* 4B) ou du partenaire végétal (riz cultivars Cigalon et Nipponbare), l'interaction conduit à la régulation d'un grand nombre de gènes, témoignant d'une réponse adaptative réciproque des partenaires l'un à l'autre (**Chapitre II, Partie1** et **Chapitre III**). Du point de vue de la bactérie, cette adaptation implique l'expression de gènes qui contribuent à la

détoxication des ROS ainsi que plusieurs systèmes codant des pompes à efflux, et ce quel que soit le cultivar. Les ROS sont produits en continu par la plante, mais leur concentration augmente en réponse à l'invasion par les microorganismes, notamment au cours des réponses de défense de type PTI et ETI (Pieterse *et al.* 2009). Si de nombreuses PGPR phytoprotectrices sont capables d'améliorer les défenses végétales en induisant l'ISR (Pieterse *et al.* 2009), l'impact des PGPR phytostimulatrices sur l'immunité des plantes a rarement été étudié. Des études réalisées sur les genres *Azospirillum* et *Burkholderia* ont montré que ces bactéries phytostimulatrices induisent des réponses de défenses moins importantes que les pathogènes (Bashan 1998 ; Bordiec *et al.* 2011). Dans le **Chapitre III** nous avons montré qu'un gène PR (Pathogen-Related) et un gène impliqué dans la synthèse des phytoalexines sont induits chez les cultivars Cigalon et Nipponbare, en réponse à l'inoculation d'*A. lipoferum* 4B ; ces gènes sont connus pour leur rôle dans les réactions de défense de type PTI et ETI (Chisolm *et al.* 2006 ; Pieterse *et al.* 2009). De nombreux gènes contenant un domaine NB-ARC, caractéristique des gènes de Résistance (gènes R) exprimés au cours de la réponse ETI (van der Biezen et Jones 1998), sont également induits. Ces résultats suggèrent que l'interaction avec la souche *A. lipoferum* 4B conduit à la mise en place d'une réponse immunitaire chez le riz. Les gènes PR, les gènes R et les phytoalexines étant induits au cours des réponses de type PTI et ETI, l'induction de telles réponses dans l'interaction *A. lipoferum* 4B-riz ne peut être exclue. Ceci pourrait se traduire par une induction de la production de ROS et expliquer pourquoi de nombreux gènes de détoxication sont induits chez les cellules d'*A. lipoferum* 4B associées aux racines (**Chapitre II**, **Partie 1**). Des réponses proches des réponses PTI et ETI sont induites au cours de la symbiose *Rhizobium*-légumineuses, conduisant par exemple à la production de ROS (Soto *et al.* 2009). Ainsi, des similitudes existent entre la perception précoce, par la plante, des bactéries pathogènes et des bactéries symbiotiques ainsi que des PGPR.

L'un des résultats les plus inattendus concernant les régulations observées pour *A. lipoferum* 4B est l'absence d'induction des gènes associés aux propriétés phytobénéfiques. La souche 4B présente deux propriétés phytobénéfiques majeures décrites à ce jour : la première implique les gènes *nif* permettant la fixation d'azote atmosphérique et la seconde concerne le gène *acdS* impliqué dans la désamination de

l'ACC, un précurseur de l'éthylène (Penrose et Glick 2003). La désamination bactérienne de l'ACC produit par les plantes pourrait conduire à une diminution de la synthèse d'éthylène dans les racines et par conséquent à une levée de l'inhibition de la croissance racinaire exercée par ce composé (Glick *et al.* 2007). Bien que plusieurs souches d'*Azospirillum* soient capables de produire de l'IAA, cette propriété n'est pas retrouvée chez la souche 4B (Wisniewski-Dyé *et al.* 2011). En revanche, elle est capable de produire une molécule induisant une réponse de type auxinique chez *Arabidopsis thaliana* (Combes-Meynet Thèse 2010). Dans les conditions testées au cours de nos travaux, aucune de ces propriétés n'apparaît significativement régulée dans les cellules d'*A. lipoferum* 4B associées aux racines de riz (**Chapitre II, Partie 1**). Ces observations sont d'autant plus surprenantes que de nombreux gènes impliqués dans le développement végétal et les voies de signalisation hormonales sont régulés chez les deux cultivars de riz en réponse à l'interaction avec *A. lipoferum* 4B. En particulier, plusieurs gènes impliqués dans la voie de synthèse d'éthylène *via* l'oxydation d'ACC ainsi que des gènes de réponse à l'éthylène sont induits, principalement chez le cultivar Nipponbare (**Chapitre III**). De plus, un nombre important de gènes associés à la synthèse et aux voies de signalisation de l'auxine sont induits chez les deux cultivars de riz. D'autres gènes impliqués dans les voies du jasmonate, des gibberellines et des cytokinines sont également régulés, témoignant d'une modulation des voies hormonales en présence de la bactérie. Concernant l'expression des propriétés phytobénéfiques d'*A. lipoferum* 4B, l'absence de modification de leur niveau d'expression au contact de la plante ne signifie pas que les gènes ne sont pas exprimés, mais seulement que la coopération ne contribue pas à leur induction par rapport à la condition témoin (ici un milieu minimum supplémenté avec 0.25 % avec du LBm). D'autre part, l'analyse réalisée ne prend en compte que le niveau moyen de l'expression des gènes le long de l'ensemble du système racinaire et la variabilité spatiale n'est pas estimée. Si l'on considère l'existence de mécanismes stochastiques qui conduisent à des niveaux d'expression variables d'une cellule à l'autre (Raj et van Oudenaarden 2008), ainsi que les variations spatiales de l'expression des gènes d'*Azospirillum* le long de la racine (Combes-Meynet *et al.* 2011 ; Vande Broek *et al.* 1993), l'existence d'inductions locales des propriétés phytobénéfiques ne peut être exclue.

L'effet d'*A. lipoferum* 4B observé conjointement sur les systèmes de défenses et sur certains gènes impliqués dans le développement est d'autant plus intéressant que chez les plantes, l'immunité et le développement sont contrôlés par des réseaux de régulation interconnectés impliquant de nombreuses hormones dont l'IAA (Pieterse *et al.* 2009). Ces interconnections permettent notamment aux végétaux de s'adapter rapidement aux variations environnementales et d'ajuster leur réponse de manière efficace en optimisant la balance coûts/bénéfices entre croissance et immunité (Pieterse *et al.* 2009). Ainsi, l'effet bénéfique des PGPR phytostimulatrices pourrait dépendre de mécanismes plus complexes que la stimulation directe de la croissance végétale généralement considérée. En effet, le déplacement direct ou indirect de l'équilibre coûts/bénéfices entre croissance et immunité, par exemple en induisant ou réprimant les défenses de la plante, pourrait contribuer indirectement aux propriétés phytostimulatrices d'*Azospirillum*.

De manière plus globale, les analyses transcriptomiques réalisées sur *A. lipoferum* 4B montrent qu'un nombre plus faible de gènes bactériens sont régulés lorsque la souche interagit avec son cultivar d'origine, le cultivar Cigalon (479), par rapport à l'interaction avec le cultivar Nipponbare (980) (**Chapitre II, Partie 1**). De même, lorsque l'on considère le partenaire végétal, un nombre plus faible de gènes est régulé chez le cultivar Cigalon (1243) que chez le cultivar Nipponbare (2141) en réponse à *A. lipoferum* 4B (**Chapitre III**). Ces résultats semblent étayer l'hypothèse de la mise en place d'une interaction préférentielle entre une souche et son cultivar d'origine et suggèrent que celle-ci pourrait conduire à un nombre plus faible de régulations génétiques. En effet, si l'on considère que des mécanismes de coévolution ont été mis en place entre une souche et son cultivar d'origine, ceux-ci pourraient avoir sélectionné préférentiellement des fonctions dont les bénéfices réciproques sont les moins coûteux pour les deux partenaires.

D'après l'ensemble des résultats discutés précédemment, nous proposons le modèle suivant afin de résumer l'adaptation des partenaires l'un à l'autre conduisant à la stimulation de la croissance du riz au cours de la coopération avec *Azospirillum* (**Figure 24**). Au-delà de l'effet direct d'*Azospirillum* sur le développement du riz (**A**), les mécanismes de co-évolution entre une souche et son cultivar d'origine pourraient

avoir conduit à une réponse de défense qui ne soit pas défavorable au développement du cultivar d'origine (**B.1**). Cet effet de la souche sur la balance coûts/bénéfices du cultivar d'origine pourrait entraîner indirectement une stimulation de la croissance (**B.2**). En retour, les défenses atténuées favoriseraient l'établissement dans la rhizosphère de bactéries (**C**), capables de contribuer davantage à la stimulation de la croissance du cultivar d'origine (**D**).

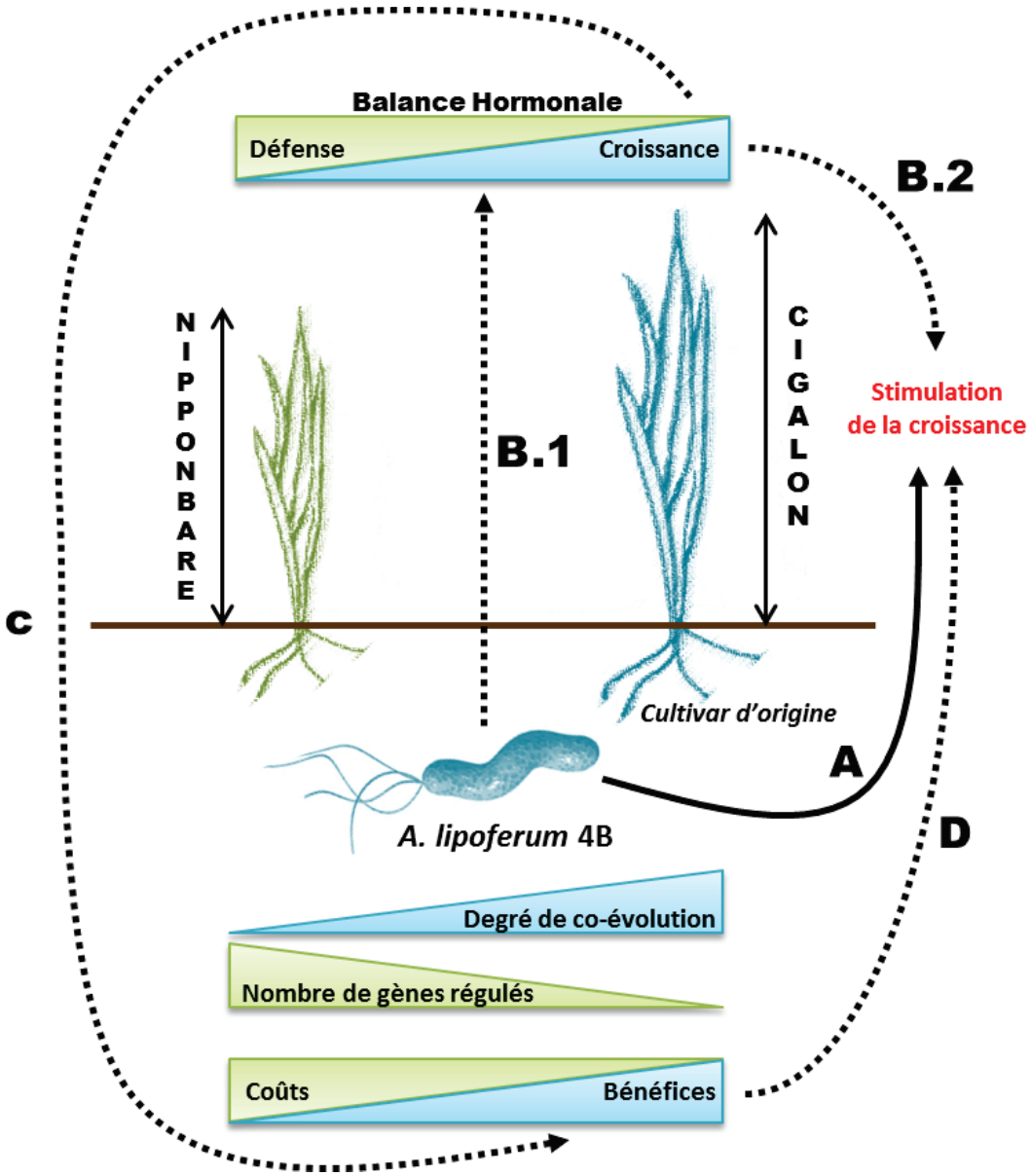

Figure 24. Modèle résumant l'adaptation des partenaires l'un à l'autre au cours de la coopération *Azospirillum*-riz.

Modification de l'expression des gènes et spécificité d'hôte

L'existence d'une spécificité d'hôte dans la coopération entre les bactéries PGPR et les plantes a été discutée dans la **Partie 2** du **Chapitre I**. Nous émettions l'hypothèse d'une spécificité d'hôte régie soit par une adaptation souche-spécifique de la bactérie à des caractères aspécifiques de la plante, soit par une adaptation aspécifique de la bactérie à des propriétés spécifiques d'un génotype de plante.

Les résultats des analyses transcriptomiques réalisées sur le couple *A. lipoferum* 4B-riz montrent que la réponse du partenaire bactérien dépend du cultivar de riz avec lequel il interagit (**Chapitre II**, **Partie 1**). Ainsi, 26 gènes ont été mis en évidence comme régulés spécifiquement au contact du cultivar Cigalon et 344 gènes ont été mis en évidence comme régulés spécifiquement au contact du cultivar Nipponbare. Une part importante de ces gènes spécifiques est impliquée dans des fonctions de régulation et de transduction du signal, témoignant d'un ajustement fin du fonctionnement des cellules bactériennes qui dépend du cultivar avec lequel elles interagissent. Plusieurs gènes impliqués dans le transport et le métabolisme des acides-aminés sont également induits de manière cultivar-spécifique. Ainsi, une part de la réponse adaptative cultivar-spécifique d'*A. lipoferum* 4B pourrait s'expliquer par des variations de la composition des exsudats entre les cultivars. Si la présence des acides aminés concernés (arginine et proline) a été mise en évidence dans les exsudats des racines de riz (Bacilio-Jiménez *et al.* 2003), à notre connaissance aucune étude n'a comparé leur concentration entre deux cultivars. Conformément à notre hypothèse, la réponse adaptative de la bactérie pourrait dépendre de propriétés spécifiques d'un cultivar.

Du point de vue du partenaire végétal, nous avons également montré que la modification de l'expression des gènes dépend de la souche bactérienne avec laquelle la plante interagit (**Chapitre III**). En effet, nous avons considéré l'impact d'une seconde souche, *Azospirillum sp.* B510, sur l'expression des gènes des cultivars de riz Cigalon et Nipponbare. Ces résultats ont été comparés à ceux observés pour *A. lipoferum* 4B et ont permis de mettre en évidence que 72 % des gènes régulés (toutes combinaisons confondues) présentent un profil d'expression spécifique d'une combinaison souche/cultivar. Ces modifications concernent des gènes impliqués dans

de nombreux mécanismes cellulaires tels que le métabolisme primaire, le métabolisme secondaire, la transduction du signal, la transcription, la traduction, le trafic intracellulaire ou encore le transport. Malgré leur rôle important dans le fonctionnement de la plante, l'étude de ces mécanismes n'a pas pu être approfondie au cours de ces travaux de thèse et leur prise en compte constitue l'une des perspectives de notre démarche d'analyse. L'objectif principal sera de comparer les régulations observées dans les voies de biosynthèse des métabolites secondaires aux variations des profils métaboliques décrites par Chamam *et al.* (sous presse). Il serait également intéressant d'approfondir l'analyse des gènes impliqués dans le métabolisme des acides aminés afin de voir si des différences existent entre les cultivars, et si ces différences peuvent expliquer l'effet cultivar-spécifique sur la régulation des gènes bactériens (voir paragraphe précédent).

De nombreux gènes impliqués dans les voies de signalisation hormonale et les réactions de défense de la plante présentent également une régulation combinaison-spécifique (**Chapitre III**). En effet, pour chaque combinaison, des voies similaires semblent engagées, comme les voies de l'auxine ou de l'éthylène, mais les gènes régulés ne sont pas les mêmes d'une combinaison à l'autre. Ceci s'observe également lorsque l'on considère les gènes potentiellement impliqués dans les systèmes de défense. De plus, si l'on considère indépendamment chaque cultivar, ces gènes sont majoritairement réprimés au cours de l'interaction avec la souche B510 alors qu'ils sont majoritairement induits au cours de l'interaction avec la souche 4B. Deux phénomènes pourraient expliquer ces variations : i) des différences dans les propriétés de modulation de la balance hormonale de chacune des souches et/ou ii) des différences dans les propriétés de colonisation racinaire. D'après l'état actuel des connaissances, les deux souches semblent présenter les mêmes propriétés phytobénéfiques, à savoir : fixation d'azote, désamination de l'ACC, et synthèse d'une molécule induisant des réponses auxiniques (Wisniewski-Dyé *et al.* 2011, 2012 **Annexe II**; Combes-Meynet Thèse 2010). En revanche, alors que la souche 4B colonise exclusivement la surface des racines de riz, la souche B510 est capable de coloniser l'interieur des tissus végétaux (Chamam *et al.* sous presse, Elbeltagy *et al.* 2001, Thomas-Bauzon *et al.* 1982). Plusieurs études tendent à montrer que dans le cas des interactions bénéfiques impliquant des bactéries endophytes, l'induction des

systèmes de défense dépend du génotype de plante et contrôle l'établissement des interactions compatibles et incompatibles (Miché *et al.* 2006 ; Rosenblueth et Martínez-Romero 2006 ; Reinhold-Hurek et Hurek 2011). Dans le cas de l'association entre l'endophyte *Herbaspirillum* et les racines de riz, la répression des thionines et d'une protéine induite par le probénazole (PBZ1) suggère que la bactérie module les défenses de la plante à son avantage (Brusamarello-Santos *et al.* 2007). En considérant ces observations, il est possible que les propriétés de la souche B510 permettant la colonisation des tissus végétaux soient associées à des propriétés d'évitement des défenses de la plante, notamment par une répression des gènes impliqués dans l'immunité. Afin d'étayer cette hypothèse, il serait intéressant de mesurer l'effet d'un plus grand nombre de souches d'*Azospirillum* sur l'expression des gènes de défense de la plante et de comparer les modifications induites par des endophytes à celles induites par des souches colonisant uniquement la surface des racines. Dans un premier temps cette démarche pourrait être entreprise par une approche de RT-qPCR ciblant des gènes de défense réprimés chez les deux cultivars en réponse à l'inoculation d'*Azospirillum sp.* B510 (voir **Chapitre III** *p137-138*).

L'ensemble de ces résultats tend à montrer que l'expression des gènes de chaque partenaire de la coopération *Azospirillum*-riz dépend à la fois de la souche bactérienne et du génotype de plante. Ces réponses transcriptomiques spécifiques s'observent à l'échelle d'une souche, d'un cultivar ou d'une combinaison souche/cultivar et soulignent la complexité des mécanismes cellulaires mis en place au cours de l'interaction entre *Azospirillum* et les racines de riz. Par ailleurs, ces résultats semblent en faveur de l'hypothèse selon laquelle la spécificité d'hôte des interactions PGPR-plantes serait régie par des réponses adaptatives réciproques qui dépendent des caractéristiques génotypiques de chaque partenaire de la coopération décrite dans la **Partie II** du **Chapitre I**.

Caractérisations fonctionnelles et perspectives.

Au-delà des enjeux techniques liés à la gestion d'une quantité importante et croissante de données, la principale limite des approches génomiques concerne la fonction des gènes étudiés. En effet, au moins 60 % des gènes d'*A. lipoferum* 4B régulés au contact de la plante codent des protéines dont la fonction est inconnue. La caractérisation fonctionnelle des gènes chez *A. lipoferum* 4B est d'autant plus compliquée que peu d'outils de mutagénèse fonctionnent. Á ce jour, seule l'inactivation de gènes par simple recombinaison homologue via le transfert conjugatif d'un plasmide non réplicatif (contenant un insert homologue au gène cible) fonctionne efficacement. Devant l'ampleur de la tâche et parmi d'autres choix possibles, nous proposons d'entreprendre l'analyse fonctionnelle de gènes potentiellement impliqués dans la transduction du signal ainsi que des régulateurs transcriptionnels. Ce choix est justifié par le fait que les bactéries du genre *Azospirillum* évoluent à l'interface entre le sol et la surface racinaire, une caractéristique qui les conduit à s'adapter continuellement aux variations des contraintes physico-chimiques ; l'écotone rhizosphérique est le siège de nombreux échanges de signaux provenant des plantes ou d'autres bactéries présentent dans la rhizosphère (**Chapitre I, Partie 1**). De plus, l'implication des systèmes de transduction du signal dans les mécanismes d'adaptation à la rhizosphère a été mise en évidence pour d'autres modèles d'interaction plante-PGPR (Matilla *et al.* 2007, Matilla *et al.* 2011).

Dans ce contexte, deux gènes impliqués dans les réseaux de régulation retiennent particulièrement notre attention. Le premier est le gène AZOLI_1541 qui est le seul gène codant une histidine kinase à être induit au contact des deux cultivars de riz (**Chapitre II**, **Partie 1**). La protéine codée par ce gène est atypique à double titre : elle contient, outre le domaine histidine kinase, un domaine receveur caractéristique des régulateurs des systèmes à deux composants (histidine kinase hybride) (Gao *et al.* 2009), mais ne contient aucun domaine transmembranaire contrairement à ce qui est généralement observé pour les histidines kinases. L'intérêt porté à ce gène est accru par le fait que parmi les génomes de bactéries interagissant avec les plantes, les génomes d'*Azospirillum* semblent posséder le plus grand nombre d'histidine kinases hybrides. Une analyse fonctionnelle permettrait donc d'identifier

le rôle et l'effecteur de cette protéine. Le second est le gène AZOLI_p10651 codant un régulateur transcriptionnel de la famille TetR qui s'avère être le gène le plus fortement régulé au contact des deux cultivars de riz, mais également au contact du blé et du maïs (**Chapitre II**). Ce gène est particulièrement intéressant car il se trouve juste en amont d'un opéron codant une pompe à efflux de type Emr-*like* qui semble également induit au contact des trois plantes. De plus, ce groupe de gènes est conservé au sein de tous les génomes d'*Azospirillum* disponibles (**Chapitre II**, **Partie 2**). L'ensemble de ces observations laissent penser que le gène AZOLI_p10651 et l'opéron en aval pourraient jouer un rôle essentiel dans la coopération entre *Azospirillum* et les cérales. Les autres gènes induits au contact des trois céréales pourraient également constituer une piste d'étude captivante ; même si bon nombre de ces gènes codent des protéines de fonction inconnue, rendant leur étude complexe, une première étape pourrait être de déterminer si leur induction peut être observée en présence d'autres plantes.

Dans un contexte plus large, il est important de considérer la rhizosphère comme un environnement dynamique, siège de nombreux échanges de signaux provenant des plantes ou d'autres bactéries rhizosphériques (**Chapitre I**, **Partie 1**). Dans le cadre de ces travaux de thèse, seules des interactions bipartites entre une céréale et une souche d'*Azospirillum* ont été prises en compte, dans des systèmes gnotobiotiques. Cette démarche a été entreprise afin de mettre en évidence les mécanismes directement impliqués dans l'adaptation des partenaires l'un à l'autre. Ainsi, elle constitue une première étape dans la compréhension des mécanismes d'adaptation potentiellement impliqués dans la spécificité des coopérations PGPR-plantes. Cependant, il sera nécessaire de valider ces observations dans des systèmes plus complexes tels qu'en sol naturel, afin de prendre en compte les interactions multipartites entre la plante, *Azospirillum* et la communauté rhizosphérique totale. La présence de microorganismes pathogènes pourrait notamment déplacer l'équilibre défense/croissance du partenaire végétal, en faveur des réactions de défense. Il serait alors intéressant d'étudier dans quelle mesure la présence d'*Azospirillum* ou d'autres PGPR phytostimulatrices contribuerait à déplacer à nouveau cet équilibre en faveur de la croissance de la plante.

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Annexes

Annexe I

Azospirillum-Plant Interaction: from Root Colonization to Plant Growth Promotion

Florence Wisniewski-Dyé, **Benoît Drogue,** Stéphanie Borland and Claire Prigent-Combaret.

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Contribution :

- Effect on Plant Growth and Health Following Inoculation with *Azospirillum*
- Agronomic Applications and Key Determinants of Future Successes

Chapter 12

Azospirillum-Plant Interaction: from Root Colonization to Plant Growth Promotion

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Introduction

Bacteria of the genus *Azospirillum* (alpha-proteobacteria) have been known for many years as plant growth-promoting rhizobacteria (PGPR) (Döbereiner and Day 1976). These free-living nitrogen-fixing highly motile bacteria can be isolated from the rhizosphere of many grasses and cereals under tropical and temperate climates (Döbereiner *et al.* 1976, Patriquin *et al.* 1983). Azospirilla are predominantly surface-colonizing bacteria, whereas other diazotrophs such as *Azotobacter diazotrophicus*, *Herbaspirillum seropedicae* and *Azoarcus* sp. are endophytic; only a few *Azospirillum* strains are able to penetrate roots (Baldani *et al.* 1986, Assmus *et al.* 1995), likely via localized enzymatic degradation of plant cell wall or via root cracks.

Azospirilla exert beneficial effects on plant growth and yield of many agronomically important crops (Okon 1985, Dobbelaere *et al.* 2001, Helman *et al.* 2011). This plant stimulatory effect has been attributed to several mechanisms, the most documented being the two main characteristics that defined the genus: production of plant growth-promoting substances (such as auxins) and biological nitrogen (N) fixation. Besides these two mechanisms, other properties are thought to be implicated in plant growth and it is likely that *Azospirillum* effect results from a combination of different mechanims (Bashan and de-Bashan 2010). Moreover, *Azospirillum* spp. can to a lesser extent enhance plant health by inhibiting plant parasites and/or by stimulating plant defence.

Azospirillum inoculation results in an increased number of lateral roots and root hairs allowing an improved uptake of minerals and water (Okon 1994, Lin *et al.* 1983, Sarig *et al.* 1988). In several developing and developed countries, notably in Latin America, *Azospirillum* is used as the bacterial inoculant of choice, alone or together with other PGPR and arbuscular mycorrhizal fungi, for many crops (Fuentes-Ramirez and Caballero-Mellado 2005, Helman *et al.* 2011, Hungria *et al.* 2010).

Among the 16 *Azospirillum* species described so far, 12 have been isolated from soil or rhizosphere: *A. amazonense* (Magalhães *et al.* 1983), *A. brasilense* (Tarrand *et al.* 1978), *A. canadense* (Mehnaz *et al.* 2007a), *A. dobereinerae* (Eckert *et al.* 2001), *A. formosense* (Lin *et al.* 2011), *A. halopraeferens* (Reinhold *et al.* 1987), *A. irakense* (Khammas *et al.* 1989), *A. lipoferum* (Tarrand *et al.* 1978), *A. melinis* (Peng *et al.* 2006), *A. oryzae* (Xie and Yokota 2005), *A. palatum* (Zhou *et al.* 2009), *A. zeae* (Mehnaz *et al.* 2007b). The two first described species, *A. brasilense* and *A. lipoferum*, remain the most studied (Baldani *et al.* 1986).

Isolation usually takes place on various N-free, semi-solid media (Bashan *et al.* 1993); an alternative technique using soil extract medium can facilitate isolation of azospirilla (Trân Vân *et al.* 1997).

This Chapter is divided into six sections dealing with: (1) features contributing to adaptation to the soil and the rhizosphere, (2) colonization of plant roots, (3) attachment to roots, (4) mechanims involved in plant-growth promotion and plant health, (5) effects on plant, and (6) agronomical applications.

Adaptation to the Soil and the Rhizosphere

Pioneer studies have shown that genomes of *Azospirillum* are constituted of multiple replicons and their sizes vary among species from 4.8 Mbp to 9.7 Mpb (Caballero-Mellado *et al.* 1999, Martin-Didonet *et al.* 2000). Recently, the complete genomes of four strains belonging to different species, isolated from various host plants and locations, were sequenced and published (Kaneko *et al.* 2010, Wisniewski-Dyé *et al.* 2011, Wisniewski-Dyé *et al.* 2012), as well as a draft genome (Sant'Anna *et al.* 2011). The size range of complete genomes is 6.5 to 7.6 Mpb and they all possess six (*A. brasilense* CBG497) or seven replicons (*A. brasilense* Sp245, *A. lipoferum* 4B, *Azospirillum* sp. B510). Whereas the biggest replicon (around 3 Mbp) has all the features of a bacterial chromosome, several replicons have been defined as chromids (Harrison *et al.* 2010); some *Azospirillum* strains appear to possess the largest number of chromids among all prokaryotic genomes sequenced to date (Wisniewski-Dyé *et al.* 2011, Wisniewski-Dyé *et al.* submitted). A clear lack of synteny was found between replicons of *Azospirillum* strains; indeed, compared to rhizobial genomes known for their genome plasticity, more genomic rearrangements have occurred in *Azospirillum* genomes and these may have been facilitated by a composite organization (Wisniewski-Dyé *et al.* 2011).

Compared to other members of the family *Rhodospirillaceae*, who live in aquatic habitats, members of the genus *Azospirillum* have the particularity of being terrestrial and plant-associated. By using a robust scheme for detecting ancestral and horizontally transferred genes in *Azospirillum*, it was shown that nearly half of the genes whose origins could be resolved appear to have been acquired horizontally from soil and plant-associated bacteria, notably from Rhizobiales and Burkholderiales (Wisniewski-Dyé *et al.* 2011). The majority of genes encoding functions critical for survival in the rhizosphere and association with plants are among those acquired horizontally: genes involved in iron acquisition (siderophore uptake and biosynthesis), transport and metabolism of compounds abundant

in plant exudates (aromatic compounds, carbohydrates and organic acids), multidrug efflux systems and osmoprotection. Elements of a type VI secretion system, also acquired laterally, may provide a means of interspecies competition to enhance environmental survival (MacIntyre *et al.* 2010).

Horizontal gene transfer was thus a major driving force in the transition of these bacteria from aquatic to terrestrial environments (Wisniewski-Dyé *et al.* 2011). These acquisitions were likely promoted by conjugation and transduction; indeed prophage regions were evidenced in *Azospirillum* genomes (Kaneko *et al.* 2010; Wisniewski-Dyé *et al.* submitted) and the release of phage particles was reported for several strains (Boyer *et al.* 2008).

The extraordinary genome plasticity disclosed by whole genome alignments has also been evidenced experimentally. Spontaneaous rearrangements between replicons were observed, leading to variants of a same strain being used in different laboratories (Pothier *et al.* 2008). Deletion or cointegration of replicons can also occur at high frequency during the process of phase variation leading to intraspecific diversity (see below). *Azospirillum* genomes carry other elements that may contribute to genome plasticity such as direct repeats, palindromic repeats, CRISPR and insertion sequences (Wisniewski-Dyé *et al.* 2011).

One of the main features of Azospirilla is to synthetize polymers such as polyhydrobutyrate (PHB), which can serve as carbon and energy source under conditions of stress or starvation (Tal *et al.* 1985, Tal *et al.* 1990). The production of PHB granules is induced when growth occurs under suboptimal conditions, such as a high C:N ratio medium (Kadouri *et al.* 2002). The ability to survive upon starvation is impaired for an *A. brasilense phbC* (encoding PHB synthase) mutant compared to the wild-type (Kadouri *et al.* 2002). Tolerance and survival to various stresses, including heat, osmotic shock, dessication, UV irradiation, and oxidative stress are reduced in the *A. brasilense phbC* mutant, as well as chemotactic responses towards attractants (Kadouri *et al.* 2003). The wild-type strain exhibits better survival than the mutant strain in carrier materials used for soil inoculants, but no difference in the ability to colonize roots and to promote plant growth was detected between the strains (Kadouri *et al.* 2003). Consequently, biosynthesis of PHB is of critical importance for improving shelf life and efficiency of commercial inoculants (Dobbelaere *et al.* 2001, Kadouri *et al.* 2003).

Unfavorable conditions, such as nutrient limitation, induce flocculation in some *A. brasilense* and *A. lipoferum* strains; cells convert to nonmotile highly refractile cyst-like forms entangled within a fibrillar matrix. The cyst-like cells contain abundant PHB granules and are surrounded by a layer of exopolysaccharides (Sadasivan and Neyra 1985). Under this dormant state, *Azospirillum* can resist desiccation, displays long-term maintenance of viability and higher heat resistance as compared with vegetative cells (Sadasivan and Neyra 1985, 1987). Interestingly, such a property has also been reported in the phylogenetic aquatic relative *Rhodospirillum centenum* (Berleman and Bauer 2004). More importantly, *A. brasilense* mutants that are impaired in cyst formation are less efficient in root colonization and nitrogenase activity (Katupitiya *et al.* 1995, Pereg-Gerk *et al.* 1998). Cyst-like *A. brasilense* cells were observed in the rhizosphere of water-stressed plants (Bashan *et al.* 1991). All these observations suggest that aggregation and encystment are of great relevance for agricultural applications.

Under cultural conditions conducive for encystment, *A. brasilense* produces compounds that form a brown pigment similar to melanin (Sadasivan and Neyra 1987, Gowri *et al.* 1996). Melanization was also observed under some circumstances in *A. lipoferum* 4B and was correlated to a laccase activity (Faure *et al.* 1994). Laccase-positive strains are less sensitive to the inhibitory action of quinone analogs due to rearrangements of their respiratory chain, a feature that might be a competitive advantage in the rhizosphere in the presence of quinone compounds (Alexandre *et al.* 1999a). A survey of bacterial laccases suggests they are an advantageous trait for a rhizosphere bacterium as they are involved in various functions such as copper resistance, manganese oxidation, pigmentation, oxidation of toxic compounds, and destruction of reactive oxygen species (Sharma *et al.* 2007).

Many azospirilla also produce carotenoids that have been shown to protect nitrogenase against oxidative damage (Hartmann and Hurek, 1988). In *A. brasilense* Sp7, the regulation of carotenoid biosynthesis involves the sigma factor RpoE; RpoE belongs to the extracytoplasmic function (ECF) sigma factors, implicated in responses to changes in the extra-cytoplasmic compartment of the cell and assumed to be critical for adaptation, survival, and growth of rhizosphere bacteria evolving in fluctuating environments. A *rpoE* deletion mutant is carotenoidless and slow-growing, and is more sensitive than the wild-type to various stresses, such as salt, ethanol and methylene blue (Mishra *et al.* 2011); moreover, a mutation in a gene encoding an anti-sigma factor causes overproduction of carotenoids in *A. brasilense* (Thirunavukkarasu *et al.* 2008).

Phase and antigenic variation is used by several bacterial species to generate intra-population diversity that increases bacterial fitness and is important in niche adaptation, or to escape host defences. By this adaptive process, bacteria undergo frequent and usually reversible phenotypic changes resulting from genetic or epigenetic alterations at specific genetic loci (Wisniewski-Dyé and Vial 2008). Several strains of *Azospirillum* can generate phenotypic variants *in vitro* and this was correlated with genome plasticity such as plasmid loss or reorganization (Vial *et al.* 2006). *A. lipoferum* 4B, a strain isolated from a rice rhizosphere, generates *in vitro* at high frequencies (10^{-4} to 10^{-3} per cell per generation) a stable phase variant named $4V_I$ exhibiting pleiotropic modifications: loss of ability to swim, to assimilate certain sugars, to reduce triphenyl tetrazolium chloride, to bind some dyes, to reduce nitrous oxide, and to deaminate 1-aminocyclopropane-1-carboxylic acid (ACC) (Alexandre and Bally 1999, Alexandre *et al.* 1999b, Prigent-Combaret *et al.* 2008). Those modifications are correlated with the loss of a 750-kb replicon (Vial *et al.* 2006). *A. lipoferum* 4T, a nonswimming strain displaying all of the features of the $4V_I$ variant, and *A. lipoferum* 4B have been isolated simultaneously from the rice rhizosphere at the same frequency (Bally *et al.* 1983); as *A. lipoferum* 4T was found to be genetically very close to *A. lipoferum* 4B, it was suggested that *A. lipoferum* 4T could in fact be a $4V_I$ variant of strain 4B generated within the soil ecosystem. After exposure of $4V_I$ to low oxygen concentrations, a laccase-positive variant can be obtained (Alexandre and Bally 1999).

Variants of *A. brasilense* Sp7 appear after exposure to prolonged starvation; those variants differ from the parental strain in several features, such as pigmentation, aggregation ability, EPS amount and composition, LPS structure and profile of outer membrane proteins (Lerner *et al.* 2010). Interestingly, one of these variants displays enhanced resistance to various stresses (Lerner *et al.* 2010). Whether phenotypic variation occurs in the rhizosphere and how it impacts survival in bacterial inoculants and plant growth promotion ability remains to be investigated.

All these key physiological properties may contribute to rhizosphere adaptation and ultimately to plant growth promotion abilities of *Azospirillum*.

Colonization of Plant Roots by *Azospirillum*

Colonization and attachment are two key processes required for a successful bacteria-plant interaction. In the rhizosphere, plant roots exude significant amounts of organic acids, sugars, amino acids and aromatic compounds. Azospirilla are highly motile and several

species exhibit a mixed pattern of flagellation: one polar flagellum is synthesized during growth in liquid medium and is primarily used for swimming whereas lateral flagella are induced during growth on solid media and are responsible for swarming over solid surfaces (Tarrand *et al.* 1978, Khammas *et al.* 1989, Moens *et al.* 1995). Motility offers the bacterium the advantage of moving towards favorable nutrient conditions. Azospirilla exhibit chemotaxis towards several root compounds, which constitutes the very early step leading to effective root colonization. Evidence supporting this affirmation was obtained by demonstrating that non-chemotactic and non-motile mutants are strongly impaired in root colonization (Vande Broek *et al.* 1998). First reports highlighting *Azospirillum* chemotactic behavior revealed that some strains exhibit positive chemotaxis *in vitro* towards several attractants such as sugar, amino acids, aromatic compounds (Reinhold *et al.* 1985, Zhulin and Armitage 1993, Lopez-De-Victoria and Lowell 1993, Alexandre *et al.* 2000) and towards root exudates (Zhulin *et al.* 1988). Migration of Azospirilla towards wheat seedlings grown in the soil was shown to be limited by soil moisture, indicating that free swimming through water films rather than swarming plays a major role in the chemotactic behavior in the rhizosphere (Bashan 1986). Interestingly, differential attraction of the bacterium, both at the species and strain levels, has been reported. As for organic acids, *Azospirillum brasilense* SpT60 (isolated from wheat rhizosphere) is strongly attracted by malate, oxalate and citrate, whereas *A. brasilense* JM6A2 (isolated from maize rhizosphere) and *A. lipoferum* ER15 (isolated from Kallar grass) do not react chemotactically to these substrates (Reinhold *et al.* 1985). These differential chemotactic responses to organic acids correlate with the exudation of these compounds by the host plant, the best attractants being generally the best growth substrates (Van Bastelaere *et al.* 1999, Alexandre *et al.* 2000). Consequently, several pieces of evidence support the existence of preferential chemotactic behavior between certain bacterial species/strains and plant species/genotypes, likely reflecting bacterial adaptation to the environmental conditions provided by the plant (Drogue *et al.* 2012).

Azospirilla also display aerotaxis, *i.e.* the directed movement towards optimal oxygen concentrations; this behavioral response can be advantageous to guide bacteria to optimal niches for nitrogen fixation (Barak *et al.* 1982). In *A. brasilense*, energy taxis, that encompasses aerotaxis, redox taxis, taxis to alternative electron acceptors, and chemotaxis to carbon sources, is the most dominant behavior (Alexandre *et al.* 2000, Alexandre *et al.* 2004). Bacteria monitor their cellular energy levels and respond to a decrease in energy by swimming to a more suitable microenvironment that can reenergize the cells. This

mechanism has the advantage of allowing migration towards environmental niches that are optimal for the bacterium metabolism (Alexandre *et al.* 2004).

Chemotaxis system integrates environment signals into an appropriate bacterial response by using a dedicated signal transduction pathway, allowing bacteria to detect changes in environmental conditions and respond by navigating towards niches optimal for growth. In *Escherichia coli*, this signal transduction system consists of a set of conserved proteins: chemotactic proteins (Che) CheA, CheW, CheY, CheB and CheR and a set of chemoreceptors known as methyl-accepting proteins (MCPs) that perceive environmental cues (Wadhams and Armitage, 2004). Upon binding a chemotactic ligand, MCPs generate chemotactic signals that are communicated to the flagellar motor via Che proteins. Among the *Azospirillum* genus, genes involved in chemotaxis have been characterized mainly in the genetically amenable species *A. brasilense*. Genes encoding a central signal transduction pathway for chemotaxis were initially identified by genetic complementation of two non-chemotactic mutants (Hauwaerts *et al.* 2002). This study has revealed the presence of *cheR* (encoding a methylesterase) and *cheB* (encoding a methyltransferase) in *A. brasilense*, thus demonstrating that responses to certain stimuli could undergo methylation and demethylation of the chemotaxis transducers, contrary to what was previously assessed (Zhulin and Armitage 1993). Although mutants lacking CheB and/or CheR homologs from this pathway are defective in chemotaxis, a mutant in which the entire chemotaxis pathway has been mutated displays a chemotaxis phenotype mostly similar to that of the parent strain, suggesting that the primary function of this pathway is not the control of motility behavior; these results also put forward the presence of multiple chemotaxis systems in *A. brasilense* (Stephens *et al.* 2006). Further characterization of this pathway (named Che1) showed that it modulates motility, cell length and flocculation and contributes indirectly to attachment to plant roots (Bible *et al.* 2008, Siuti *et al.* 2011). Recently, with the availability of four complete *Azospirillum* genomes, the existence of multiple chemotaxis systems has been confirmed (Wisniewski-Dyé *et al.* 2011). Indeed, the four strains host three chemotaxis systems of ancestral origin (including Che1), but they also have horizontally acquired up to three more chemotaxis operons resulting in four, five and six chemotaxis systems in *A. brasilense* Sp245, *A. lipoferum* 4B and *Azospirillum* sp. 510, respectively (Wisniewski-Dyé *et al.* 2011). The biological role of these different chemotaxis operons is not clear, but may fine-tune behavioural responses to different environmental conditions, and provide another level of control of the motile chemosensory behavior (Alexandre *et al.* 2004). Several MCPs have been identified in *A. brasilense*: Tlp1 involved in energy taxis and in colonization of plant roots (Greer-Phillips *et al.* 2004) and AerC functioning as a redox sensor (Xie *et al.*

2010). ChsA, displaying a PAS sensory domain and an EAL transmitter domain, was recently shown to be involved in chemotaxis as a partially reduced chemotactic response could be observed in a *chsA* mutant (Carreño-Lopez *et al.* 2009). The search for plant-inducible genes from *A. brasilense* led to the identification of a sugar-binding protein SpbA, specifically induced by wheat root exudates. Further characterization revealed that SpbA is involved in *A. brasilense* chemotaxis response towards D-galactose, L-arabinose and D-fucose (Van Bastelaere *et al.* 1999). This protein is very similar to ChvE of *Agrobacterium tumefaciens*, a periplamic component of a sugar ABC transporter; this suggests that the first steps of rhizobacteria-plant interaction are conserved among different bacteria.

As well as for chemotactic signaling and molecular mechanisms governing chemotaxis, the ecological role of this complex behaviour is not yet fully understood. Nonetheless it must provide significant advantage to rhizobacteria that must perpetually seek for optimal growth conditions and compete with other microorganisms in such a stochastic environment constituted by the rhizosphere. Unraveling the genetic determinants involved in chemotactic response may represent an initial step in selecting *Azospirillum* as inoculants of different crops.

Attachment to Roots

Attachment of bacteria to plant roots is an important step for the establishment of an effective bacteria-plant association. A study of inner-root colonization of wheat by *A. brasilense* Sp7 revealed discrepancies from one cultivar to another, evidencing preferential PGPR/plant pair associations, even if other pairs were effective (Mostajeran *et al.* 2007). Similarly, bacterial strain/plant genotype affinities were evidenced, with *A. brasilense* cells adhering more efficiently to the cultivar from which they had been isolated (Egorenkova *et al.* 2000). Preferential colonization sites have been determined by using direct microscopy examination of the bacterium on the root surface. By monitoring the colonization pattern of *A. brasilense* Sp245 constitutively expressing the *gusA* gene, it was observed that the sites of primary root colonization are the root hair zones and the site of lateral root emergence, but not the older part of the root system (Vande Broek *et al.* 1993). Interestingly, colonization of the root surface by the bacterium involves a morphological change reminiscent of the morphology of cysts. Visualization of rice root colonization by two *Azospirillum* species reveals that colonization sites depend on the species; indeed, while *A. irakense* cells are mainly associated with rice root hairs, *A. brasilense* cells are mainly located on root surfaces (Zhu *et al.* 2002). These different colonization patterns were attributed to bacterial shapes

(vibroid for *A. irakense*, cyst-like for *A. brasilense*), which probably are an adaptation to their natural environment (wetland for *A. irakense* and dry land for *A. brasilense*). Moreover, *Azospirillum* displays strain-specific differences in the way they colonize roots. *In situ* monitoring with fluorescently labelled rRNA-targeted oligonucleotide probes in combination with scanning confocal microscopy revealed that *A. brasilense* Sp245 forms microcolonies in the intercellular spaces of the inner root tissue of wheat, while *A. brasilense* Sp7 is restricted to the root hair zone (Assmus *et al.* 1995). These differences in spatial distribution suggest the existence of specificity between bacterial strain surface components and some plant receptors on the root surface (Drogue *et al.* 2012).

Two different modes of attachment of *Azospirillum* have been proposed: i) a rapid, reversible and weak adsorption mediated by proteins, and ii) a strong and irreversible anchoring mediated by bacterial extracellular polysaccharides (Michiels *et al.* 1991, Croes *et al.* 1993). Several bacterial components have previously been described as important features for root attachment but the exact mechanism involved has not been described in full details yet. In *A. brasilense*, a major outer membrane protein was shown to act as an adhesin, and may be consequently involved in bacterial aggregation and adsorption to plant roots (Burdman *et al.* 2000a, 2001). The polar flagellum also mediates the initial adsorption step. Indeed, purified polar flagellum of *A. brasilense* binds to wheat root, whereas lateral flagella do not (Croes *et al.* 1993). Further characterization revealed that the polar flagellum Fla1 is a glycoprotein which can mediate adsorption to roots (Moens *et al.* 1995). The TAD (tight adherence) secretion system is another structural component essential for host-colonization and biofilm formation in numerous other bacteria genera (Tomich *et al.* 2007). Analysis of the *A. brasilense* Sp245 genome revealed the presence of genes encoding TAD pili. A mutant deficient in TAD pili had a severe defect in adhesion to abiotic surface (Wisniewski-Dyé *et al.* 2011). Thus, TAD pili could also play a role in the bacterium attachment process but its role in *Azospirillum*-plant colonization needs further investigation.

The second step, root anchoring, is mediated by extracellular components exposed to plant root surface, such as expolysaccharides (EPS), capsular polysaccharides (CPS), lipopolysaccharides (LPS). In *A. brasilense*, a mutant deficient in EPS was shown to be impaired in root anchoring, suggesting a strong role of EPS in root attachment (Michiels *et al.* 1991). Moreover, an *A. brasilense* pleiotropic mutant, displaying a thicker layer of EPS than the wild-type, is defective in aggregation process (Blaha and Schrank 2003). A gene encoding for a LuxR transcriptional regulator, termed *flcA*, controls exopolysaccharides

production, flocculation and wheat root colonization in *A. brasilense* Sp7 (Pereg-Gerk *et al.* 1998). EPS and CPS composition vary among different *Azospirillum* strains, and is correlated with the ability of these strains to aggregate *in vitro* (Burdman *et al.* 2000b). The aggregation ability of different *A. brasilense* strains depends on both the concentration and composition of expolysaccharides (Burdman *et al.* 1998, 2000b, Bahat-Samet *et al.* 2004). Among five of the main monosaccharides found in the extracellular bacterial cell surface, L-arabinose is able to strongly inhibit *in vitro* aggregation and none of this monosaccharide could be detected in *A. brasilense* strains defective in aggregation, providing evidence for the involvement of L-arabinose in aggregation (Jofré *et al.* 2004, Bahat-Samet *et al.* 2004).

As previously described in other plant-bacteria interactions, there are several pieces of evidence showing that lectins are implicated in root attachment (Umali-Garcia *et al.* 1980). Several strains of *A. lipoferum* and *A. brasilense* can bind the lectin wheat germ agglutinin (WGA), suggesting the presence of sugar-binding receptors for WGA on the bacterial cell surface (Del Gallo *et al.* 1989). In *A. lipoferum*, a 32 kDa capsular glycoprotein was found to be the WGA-ligand (Karpati *et al.* 1999). Surface attachment of *A. brasilense* can be increased by nitrogen limitation and can be promoted *in vitro* by lectins, suggesting that it depends on interaction with surface-exposed residues within the extracellular matrix of cells (Siuti *et al.* 2011). In addition, the occurrence of cell-surface lectins by several *Azospirillum* species was demonstrated (Castellanos *et al.* 1998). More particularly, *A. brasilense* Sp7 produces a 67 kDa outer-membrane lectin that can bind surface-exposed extracellular polysaccharides of other bacterium (Mora *et al.* 2008). Thus, it was suggested that bacterial lectins could also play a role in cell-to-cell adhesion and subsequent aggregation to plant root, altogether leading to effective root colonization.

As occurs in several plant-bacteria associations, LPS are also involved in the attachment process to roots by *Azospirillum. A. brasilense* LPS are composed of glucose, galactose, xylose, rhamnose, fucose and glucosamine, whereas those of *A. lipoferum* contain mainly glucose (Jofré *et al.* 2004, Vanbleu *et al.* 2005). The O-antigen chains of LPS from several *Azospirillum* strains are composed of linear pentasaccharide repeats containing only D-rhamnose residues (Konnova *et al.* 2008). In *A. brasilense* Cd, the disruption of the *rmlD* gene involved in rhamnose biosynthesis lead to a pleiotropic phenotype; the *rmlD* mutant displays altered LPS core structure, an increased EPS production and colonizes maize roots to a lesser extent than the wild-type (Bahat-Samet *et al.* 2004, Jofré *et al.* 2004). However, no LPS plant receptor has been evidenced yet.

Attachment of *Azospirillum* to plant roots undoubtedly acts as a key factor in determining bacterial competitiveness to colonize the root. Even if the precise mechanism has not been discovered so far, efficient adhesion to plant cells seem to be a complex interplay between *Azospirillum* cell surface components and plant root receptors. The well-adapted phenotype of azospirilla to the rhizosphere of their host plant is actually a prerequisite for its growth promoting effects.

Mechanisms Involved in Plant Growth Promotion and Plant Health

Direct Stimulation of Plant Growth

Most *Azospirillum* strains are able (i) to stimulate plant nutrition through nitrogen fixation, phosphate solubilisation, or through their impact on plant root system architecture, and (ii) to enhance plant growth through production of phytohormones (Table 1).

Azospirillum is a free-nitrogen fixing bacterium which had long time been isolated on the basis of its positive response to acetylene reduction assays. Several reports evidenced that following *Azospirillum* inoculation, there is a significant increase in the total N in shoots but only part of fixed N by the bacterium appeared to be transferred to the inoculated plant (Kapulnik *et al.* 1981). Thus, many studies showed that the contribution of N_2 fixation by *Azospirillum* to the plant represents less than 20% of the total N increase in the plant. Mutants deficient in nitrogenase activity (*i.e.* Nif mutants) have been shown in several cases to retain their ability to promote plant growth of certain crops (Bashan *et al.* 1989). This questions the relative contribution of N_2 fixation to the growth promotion effect. In contrast to symbiotic N_2 fixation, where there is direct transfer of N across the symbiotic interface, *Azospirillum* like other root surface associated diazotrophs seems not able to readily release fixed N to the host plant and this process occurs mainly through microbial turnover (Lethbridge and Davidson 1983, Rao *et al.* 1998).

Azospirillum can solubilize phosphate through the release of gluconic acid such as in *A. brasilense* and *A. lipoferum* or through uncharacterized mechanisms (Bashan and de-Bashan 2010, Puente *et al.* 2004, Ramachandran *et al.* 2007, Seshadri *et al.* 2000). The ability of *Azospirillum* to enhance plant uptake of key minerals like nitrates, ammonium, potassium, iron and several metallic micro-nutrients has been reported (Barton *et al.* 1986, Lin *et al.* 1983, Murty and Ladha 1988, Ogut and Er 2006).

Table 1: Proposed modes of action of *Azospirillum* on plants, besides nitrogen fixation.

Mode of action	Specific mechanism	References
Production of phytohormones	Abscisic acid	Cohen *et al.* 2008, 2009
	ACC deaminase activity	Glick *et al.* 2007a, 2007b Prigent-Combaret *et al.* 2008
	Cytokinins	de García Salamone *et al.* 2001 Perrig *et al.* 2007 Tien *et al.* 1979
	Ethylene	Perrig *et al.* 2007 Ribaudo *et al.* 2006 Thuler *et al.* 2003
	Gibberellins	Bottini *et al.* 1989, 2004 Cassán *et al.* 2001, 2009a Perrig *et al.* 2007 Piccoli *et al.* 1997, 1999
	IAA	Barbieri and Galli 1993 Carreño-Lopez *et al.* 2000 Dobbelaere *et al.* 1999 Fallik *et al.* 1989 Malhotra and Srivastava 2008 Prinsen *et al.* 1993 Spaepen *et al.* 2007b Vande Broek *et al.* 1999
	NO	Creus *et al.* 2005 Molina-Favero *et al.* 2008 Pothier *et al.* 2007
	Other auxins	Costacurta *et al.* 1994 Fallik *et al.* 1989 Hartmann *et al.* 1983 Somers *et al.* 2005
Production of polyamines		Cassán *et al.* 2009b Perrig *et al.* 2007
Solubilization of phosphate		Puente *et al.* 2004 Ramachandran *et al.* 2007 Seshadri *et al.* 2000
Biological control	ACC deaminase activity	Toklikishvili *et al.* 2010
	Competition with bacterial pathogens	Bashan and de-Bashan 2002
	Induced Systemic Resistance	Yasuda *et al.* 2009
	Inhibition of germination of parasitic weeds	Bouillant *et al.* 1994 Dadon *et al.* 2004 Miché *et al.* 2000
	Production of cyanidric acid	Gonçalves and de Oliveira 1998
	Production of bacteriocins	Oliveira and Drozdowicz 1987 Tapia-Hernández *et al.* 1990
	Production of siderophores	Shah *et al.* 1992 Tapia-Hernández *et al.* 1990

Azospirillum spp. was shown to modulate the plant hormonal balance, particularly by producing a wide diversity of plant hormones, *i.e.* auxins, gibberellins (GAs), cytokinins, and ethylene that have different effect on plant root system architecture. The production of phytohormones by *Azospirillum* mostly enhances root branching, resulting in a greater surface of the root system. Thus, plant roots can explore a wider soil volume and thereby improve the mineral and aqueous nutrition of the plant. Phytohormone production rather than N_2 fixation is considered as the main way *Azospirillum* promotes plant growth (Bashan and de-Bashan 2010, Bottini *et al.* 2004, Spaepen *et al.* 2007a). But, even if transfer of hormones from bacteria to the plant might occur, it has been rarely evidenced *in vivo*, during the course of the associative symbiosis.

Indole-3-acetic acid (IAA) is the best characterized auxin produced by PGPR including *Azospirillum* strains. It controls a wide variety of processes in plant development and plant growth and plays a key role in shaping plant root architecture such as regulation of lateral root initiation, root vascular tissue differentiation, and polar root hair positioning (Aloni *et al.* 2006, Fukaki *et al.* 2007). IAA is usually synthesized from tryptophan, which can be found by PGPR in root exudates at different concentrations according to the plant genotype (Kamilova *et al.* 2006). However, in *Azospirillum*, a tryptophan-independent pathway has been reported (Carreño-Lopez *et al.* 2000; Prinsen *et al.* 1993). The known routes of tryptophan-dependent IAA biosynthesis in *Azospirillum* includes: IAA formation via (i) indole-3-pyruvic acid (IPyA), (ii) indole-3-acetonitrile (IAN), (iii) tryptamine or (iii) indole-3-acetamide (IAM) formation (Baca *et al.* 2003, Carreño-Lopez *et al.* 2000, Zakharova *et al.* 1999, Kaneko *et al.* 2010, Wisniewski-Dyé *et al.* 2012). The indole pyruvate decarboxylase, encoded by the *ipdC/ppdC* gene, is a key enzyme in the IPyA pathway that mediates conversion of IPyA into indole-3-acetaldehyde. Various *ipdC* mutants from *Azospirillum* spp. displayed altered phenotypes compared to the wild type strains in their ability to alter plant root morphology (Dobbelaere *et al.* 1999, Malhotra and Srivastava 2008). But, due to the redundancy in IAA biosynthesis pathways, most *ipdC* mutants were shown to still produce around 10% of the wild-type IAA production level, and to retain the property of modifying root system architecture (Barbieri and Galli 1993, Prinsen *et al.* 1993, Spaepen *et al.* 2007b, Vande Broek *et al.* 1999). The involvement of IAA production in plant growth promotion by *Azospirillum* was also evidenced by increased IAA levels observed in inoculated maize plants (Fallik *et al.* 1989). Besides IAA production, *Azospirillum* seems to produce several other key auxin-type molecules like indole butyric acid, phenyl acetate, indole acetaldehyde, or indole acetamide (Costacurta *et al.* 1994, Fallik *et al.* 1989, Hartmann *et al.* 1983, Somers *et al.* 2005).

Production of bacterial gibberellins, which corresponds mostly to GA1, GA3 and GA4 has been reported in *Azospirillum lipoferum* (Perrig *et al.* 2007). Gibberellins enhance the development of plant tissues, particularly stem tissue, promote root elongation and extension of lateral roots and are involved in breaking dormancy during seed germination (Barlow *et al.* 1991, Yaxley *et al.* 2001). A combination of both gibberellin production and hydrolysis of glucosyl-conjugates of gibberellic acid, which correspond to storage or transport forms of gibberellic acid produced by plants, occurs in *Azospirillum* (Cassán *et al.* 2001, Piccoli *et al.* 1997, 1999). Production of GA by *Azospirillum* has been shown to be involved in growth promotion of maize (Lucangeli and Bottini 1997), and to improve maize and soybean seed germination (Cassán *et al.*, 2009a). However, the bacterial genetic determinants involved in this mechanism remain to be identified, as does the precise role of gibberellins in plant growth promotion by PGPR.

Cytokinin production (especially zeatin) has been documented in *Azospirillum brasilense* (de García Salamone *et al.* 2001, Perrig *et al.* 2007, Tien *et al.* 1979). Cytokinins stimulate plant cell division, control root meristem differentiation, induce proliferation of root hairs, whereas they inhibit lateral root formation, and primary root elongation (Riefler *et al.* 2006, Silverman *et al.* 1998). However, the real contribution of cytokinin production by PGPR to plant growth promotion is rather speculative so far, because genetic determinants genes involved in putative cytokinin bacterial biosynthetic pathways were only identified by comparative genomics and their role has not been validated by functional studies (Frébort *et al.* 2011).

Bacterial production of abscisic acid (ABA), which is involved in plant stress alleviation, has been less studied. The ability of bacterial ABA to promote plant growth has been documented in *A. lipoferum* and *A. brasilense*, but a positive effect has been only observed in water-stressed plants (Cohen *et al.* 2008, 2009).

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Ethylene is another key phytohormone produced in small amounts by some *A. brasilense* from methionine as a precursor (Perrig *et al.* 2007, Thuler *et al.* 2003). It inhibits root elongation and auxin transport, and promotes seed germination, senescence and

abscission of various organs and fruit ripening (Bleecker and Kende 2000, Glick *et al.* 2007b). Ethylene is also involved in plant defense pathways (Glick *et al.* 2007b). The ability of *A. brasilense* to produce ethylene seems to promote root hair development in tomato plants (Ribaudo *et al.* 2006). However, to evaluate the contribution of ethylene in *Azospirillum* growth promoting effect, characterization of the biosynthetic pathway and genetic determinants is required. On the contrary, certain *Azospirillum* strains especially from the *lipoferum* species are able to lower plant ethylene levels through deamination of ACC. ACC deaminase (encoded by the *acdS* gene) catalyses the conversion of ACC, the immediate plant precursor for ethylene, into NH_3 and α-ketobutyrate. Since ethylene inhibits growth and elongation of root, this may lead to enhanced root system development (Glick *et al.* 2007a). In the case of *A. brasilense* strains, complementation of $AcdS^-$ strains with an *acdS* gene from *P. putida* enhanced the plant-beneficial effects of these PGPR on both tomato and canola (Holguin and Glick 2001, 2003).

Polyamines are considered as plant growth regulating compounds; among them, cadaverine production was evidenced in some *Azospirillum* strains (Cassán *et al.* 2009b, Perrig *et al.* 2007) and its possible role in rice plant growth promotion and osmotic stress mitigation was reported (Cassán *et al.* 2009b). Production of vitamins was also reported for *Azospirillum* but whether this trait is involved in plant growth-promotion remains to be investigated (Rodelas *et al.* 1993).

Finally, some *Azospirillum* strains like *A. brasilense* Sp245 are able to produce nitric oxide (NO), during root colonization, due to the activity of nitrite reductases (Creus *et al.* 2005, Molina-Favero *et al.* 2008, Pothier *et al.* 2007). Increasing evidence indicates that NO is a key signalling molecule involved in a wide range of effects on plants, including control of the formation of lateral and adventitious roots (Creus *et al.* 2005, Molina-Favero *et al.* 2008).

Biocontrol Properties of *Azospirillum*

Mostly described for bacteria of the genus *Pseudomonas*, the ability of some *Azospirillum* strains to protect plant from parasites has been shown for more than 20 years; this enhanced resistance against diseases caused by virulent fungus and bacteria relies on different mechanisms such as (i) inhibition or retardation of phytopathogens growth by competition or production of antimicrobial compounds, (ii) inhibition of parasitic weeds, like *Striga hermontica*, or (iii) enhancement of plant resistance to pathogen infection.

Besides its direct plant growth promoting effects, *A. brasilense* can inhibit *Agrobacterium*-induced crown gall, bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato* or bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* on tomato (Bakanchikova *et al.* 1993, Bashan and de-Bashan 2002, Romero *et al.* 2003). The biocontrol effect towards *Agrobacterium* seems to be correlated with the ability to deaminate ACC (Toklikishvili *et al.* 2010). Prevention of bacterial speck disease development by *A. brasilense* may be the consequence of plant growth promotion leading to a more robust plant, or competition between *Azospirillum* and the pathogen (Bashan and de-Bashan 2002). *Azospirillum* also protects plants against fungus pathogen attacks, such as *Rhizoctonia* spp. and *Colletotrichum acutatum* (Russo *et al.* 2008, Tortora *et al.* 2011). Reduction of anthracnose symptoms, observed when *A. brasilense* is inoculated on strawberry plants 15 days before the infection with the fungus *Colletotrichum acutatum*, coincides with the ability of *Azospirillum* strains to produce siderophores displaying antifungal effects *in vitro* (Tortora *et al.* 2011). All these observed antimicrobial activities could be related to the ability of *Azospirillum* spp. to produce bacteriocins, siderophores, cyanidric acid, and phenylacetic acid (Gonçalves and de Oliveira 1998, Oliveira and Drozdowicz 1987, Shah *et al.* 1992, Somers *et al.* 2005, Tapia-Hernández *et al.* 1990, Tortora *et al.* 2011).

Rice inoculated with the endophyte *Azospirillum* sp. B510, display significant resistance against rice blast disease caused by the fungus *Magnaporthe oryzae* and rice blight disease caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (Yasuda *et al.* 2009). Interestingly, pathogenesis-related genes and salicylic acid accumulation are not induced, suggesting that the priming effect of *Azospirillum* endophytes may involve a novel type of plant resistance mechanism, independent of salicylic acid signaling.

Several strains of the *Azospirillum* genus can inhibit the parasitic plant *Striga* by stopping radicle development of GR24-germinated *Striga* seeds (Bouillant *et al.* 1997, Miché *et al.* 2000). The ability of a low-molecular-weight alcohol-soluble compound of *A. brasilense* to inhibit the germination and radicle growth of GR24-conditioned seeds of *Orobanche aegyptiaca* was also reported (Dadon *et al.* 2004).

It must be noticed that the production of both antimicrobial (biocontrol) and plant growth-promoting compounds, implicated in the functioning of the associative symbiosis, appears to be strain-specific (Tortora *et al.* 2011, Perrig *et al.* 2007). Many determinants involved in plant growth-promoting effect, at least those characterized, are among those

acquired horizontally (Wisniewski-Dyé *et al.* 2011). Such acquisitions may have occurred before or after speciation events.

Moreover, expression of the corresponding genes might be modulated by rhizosphere signals, some of them being produced by other members of the microbial community (Combes-Meynet *et al.* 2010), some being specifically produced by peculiar plant genotypes (Drogue *et al.* 2012, Drogue *et al.* In press). One of the best documented examples is the influence of root exudates composition on phytohormone production (Ona *et al.* 2006, Rothballer *et al.* 2005). Thus, regulation of phytohormone production by PGPR might constitute an important component of the specificity in the plant-*Azospirillum* associative symbiosis.

Effect on Plant Growth and Health Following Inoculation with *Azospirillum*

Initially described as a cereal growth promoter, it has become clear that the beneficial effects of *Azospirillum* are not limited to growth promotion and not even to cereal crop plants. More than 100 plant species, including both monocots and dicots, were successfully inoculated with *Azospirillum* or consortia of microorganisms containing *Azospirillum* (Bashan *et al.* 2004). In most cases successful inoculation results in (i) roots and shoots morphological changes, (ii) yield enhancements, and/or (iii) nutrition improvements. Among the reported morphological changes, root and shoot length are currently investigated. Other parameters such as root and leaf area, root diameter, root hair development and number of root tips were also reported to be modified. Yield enhancements result in increases in grain weight, number of grains per plant, germination rate and biomass. When considering plant nutrition, improvements in nitrogen, phosphorus, potassium, sugar and chlorophyll contents were noticed.

During the last 30 years, plant growth promotion was mainly studied on wheat, maize and rice, probably because most of the *Azospirillum* strains were initially isolated from cereals (Bashan *et al.* 2004, Fuentes-Ramirez and Caballero-Mellado 2005, Veresoglou and Menexes 2010). However, an increasing number of strains are isolated from non-cereal plants, leading scientists to consider the beneficial effects of *Azospirillum* on a wider range of ornamental and agricultural plants. Thus, strawberry plants, sugarcane and blanket flower were reported to be natural hosts for *Azospirillum* (Pedraza *et al.* 2007, Gadagi *et al.* 2004, Tejera *et al.* 2005). Natural isolates of *A. brasilense* inoculated on strawberry plants induce significant changes in root morphology, resulting in enhancements or decreases in

root length and dry weight (Pedraza *et al.* 2009). These changes are also observed on shoot dry weight, which emphasizes the importance of rhizospheric interactions in health and yield of the whole plant. Similar results were reported on the ornamental plant *Gaillardia pulchella*, as natural isolates promote plant height, number of leaves and branches as well as dry matter of the whole plant (Gadagi *et al.* 2004). Interestingly, these studies point out that both plant and *Azospirillum* genotypes are determining factors for the establishment of a successful interaction between PGPR and plants. Such observations were already made on cereals and especially on rice. In a study where 37 isolates of *Azospirillum* spp. were inoculated on lowland rice, 5 isolates enhanced plant height and root and shoot dry weights, 20 isolates promoted only root and shoot dry weights, 9 isolates enhanced only shoot dry weight and 3 isolates showed no beneficial effect (Gunarto *et al.* 1999). Similarly, stem height, stem dry matter, root length and N accumulation of rice differ according to the isolate of *A. amazonense* that was inoculated (Rodrigues *et al.* 2008).

From the earliest field experiments with *Azospirillum* in the 1980s, the most striking effects on plant growth and yield were obtained when the growth conditions were suboptimal. Mitigation of salt stress was observed on maize and wheat (Bacilio *et al.* 2004, Creus *et al.* 1997, Hamdia and El-Komy 1997, Hamdia *et al.* 2004); in maize, bacteria seem to restrict Na^+ uptake, enhance the uptake of K^+ and Ca^{2+}, and stimulate nitrate reductase activity (Hamdia *et al.* 2004). Inoculation with *Azospirillum* also improves growth under drought conditions, as demonstrated for sorghum and wheat (Creus *et al.* 2004, El-Komy *et al.* 2003, Sarig *et al.* 1990); this could result from better water uptake as a response to inoculation. *Azospirillum* inoculation slightly enhances root length and biomass of barley seedling treated with cadmium and the amount of nutrients absorbed by the inoculated plants increases significantly, demonstrating that *Azospirillum* can also mitigate metal toxicity (Belimov and Dietz 2000). So far, ABA and cadaverine were reported to be involved in stress mitigation (Cassán *et al.* 2009b, Cohen *et al.* 2009) but alleviation of these stresses may operate via other mechanisms that remain to be characterized.

Recently, a new facet of the interaction between *Azospirillum* and cereals was explored, *i.e.* its impact on secondary metabolism of the host plant (Walker *et al.* 2011). In addition to shoot biomass enhancement, metabolites profiles of maize are modified at a qualitative and quantitative level, depending on the inoculated strain and the maize cultivar. Interestingly, the modification is strain-specific and differences at the strain level are more important than differences between bacterial species or geographic origins. Indeed, the inoculation of *A. brasilense* CFN-535 (from Mexico) and *A. lipoferum* CRT1 (from France)

triggers similar profiles, whereas *A. brasilense* UAP-154 (from Mexico) inoculation triggers a different one. Among the compounds showing quantitative changes, individual benzoxazinoids may be important for plant interactions with bacteria, and they may serve as early markers of effective PGPR-maize interactions (Walker *et al.* 2011).

Among all the unresolved issues about associative symbiosis between *Azospirillum* and plants, the less addressed is that of interaction specificity. On one hand, some studies point out a lack of specificity (Isawa *et al.* 2010, Naiman *et al.* 2009, Puente and Bashan, 1993). On the other hand, studies described above underline the importance of plant and *Azospirillum* genotypes in the establishment of an effective interaction (Gadagi *et al.* 2004, Pedraza *et al.* 2009, Rodrigues *et al.* 2008, Walker *et al.* 2011). Then, genotypes must be taken into account when considering direct plant growth promotion of *Azospirillum* and other PGPR (Moutia *et al.* 2010). Interestingly, examples of both demonstrated specificity and known mechanisms of recognition that suggest specificity can be underlined for each step of the accomplishment of an effective associative symbiosis, *i.e.* chemotactic attraction of *Azospirillum*, root colonization, production of antimicrobial (biocontrol) and plant growth-promoting compounds (see above, Drogue *et al.* 2012). However, the lack of studies including a large range of host genotypes and bacterial strains prevents a clear appraisal of the level of specificity in *Azospirillum*-plant interactions.

Agronomic Applications and Key Determinants of Future Successes

The agronomic potential of bacteria has been studied for long, based on farmers' experience, and plant inoculation with *Rhizobium* sp. became common since the end of the 19th century (Bashan 1998, Nobbe and Hiltner 1896). It was not until the 1930s that PGPR, mainly *Azotobacter* and *Bacillus*, were used on large scale field experiments in Europe, with rather unsatisfying results (Macdonald 1989). Plant growth-promoting abilities of *Azospirillum*, highlighted in the mid-1970s, revived the interest of PGPR for plant inoculation, mainly on economically important non-legume plants (Dobereiner and Day 1976).

During the last 40 years, *Azospirillum* effects on plant growth were extensively studied, and the proportion of field experiments is increasing, due to economic and environmental benefits of biofertilizers. Most of the studies were conducted in India and Latin America where several *Azospirillum* inoculants are now commercialized (Bashan *et al.* 2004, Fuentes-Ramirez and Caballero-Mellado 2005). The analysis of data accumulated during the 1980s and 1990s revealed that *Azospirillum* inoculation induces statistically significant yield improvements, ranging from 5% to 30%, in 60-70% of the experiments

(Okon and Itzigsohn 1995). Evaluations made by farmers and agronomists in a large field experiment carried in 1999 in Mexico, indicated that inoculated maize, sorghum and barley display mean grain yield enhancements of 8%, 11.5% and 63.5% respectively (Fuentes-Ramirez and Caballero-Mellado 2005). A recent survey conducted on data published from 1981 to 2008, evaluated the impact of *Azospirillum* inoculation on wheat growth and yield (Veresoglou and Menexes 2010). It appears that grain yield is increased by 8.9% and aboveground dry weight by 17.8% on average, when *Azospirillum* is inoculated on wheat. Despite these numerous consistent field results, the commercialization of *Azospirillum* biofertilizers remains limited, due to the observed variability, and to the lack of consideration of key determinants for the plant growth-promoting effect.

Many field experiments have been carried out without considering the importance of climate, soil, host plant, mineral fertilization and *Azospirillum* strain combinations. Meta-analysis of experiments conducted on wheat, points out that (i) applied N fertilization, (ii) plant cultivar and (iii) bacterial strains are key determinants for the success of plant growth promotion (Veresoglou and Menexes 2010). Thus, inoculations of wheat are more efficient when no N fertilization is applied. Similar observations were made on barley as yield increases, associated with *Azospirillum* inoculation, diminished when fertilizers are applied at high levels (Ozturk *et al.* 2003). Whereas the inoculation of *Azospirillum* positively impacted grain yield of maize in 93% of the non fertilized sites, beneficial effects were observed in only 50% of the highly fertilized sites (Fuentes-Ramirez and Caballero-Mellado 2005). So, rational application of fertilizers and bacterial inoculants may generate savings of chemical fertilizers. Some authors suggested that these reductions could be around 20% to 50% (Bashan et al 2004, Okon and Labandera-Gonzalez 1994). Moreover, taking into account the importance of plant and bacterial genotypes in the establishment of an effective interaction (see above) and exploring interaction specificity in the associative symbiosis, inoculants containing *Azospirillum* will be more effective in the future. However, it is clear that a particular strain cannot be universally successful, in any soil, with any host plant and agricultural practices (Fuentes-Ramirez and Caballero-Mellado 2005).

A major progress in the early 1990s was the co-inoculation of *Azospirillum* with other microorganisms (Bashan *et al.* 1998). Inoculation of consortia conducted to more successful and more beneficial impacts on plant health and growth (Bashan *et al.* 2004). When *Azotobacter* and *Azospirillum* are co-inoculated on wheat, beneficial effect on seed yield seems to be further increased than for single inoculations (Veresoglou and Menexes 2010). Similarly, nitrogen and phosphorus concentrations in tomato, red pepper and rice are

higher when *Methylobacterium oryzae* and *A. brasilense* are co-inoculated (Madhaiyan *et al.* 2010). The combination of the fungus *Trichoderma* and *Azospirillum* can improve bean seed yield if mineral phosphate is applied at one ton per ha (Ogut *et al.* 2005). *A. brasilense* also stimulates the development of vesicular arbuscular mycorrhiza, notably on maize (Barea *et al.* 1983, Ratti *et al.* 1996). Interestingly, *Azospirillum* is also described to be a *Rhizobium*-"helper", as several studies reported positive effects of *Azospirillum*/*Rhizobium* combinations (Delgallo and Fabbri 1991, Dardanelli *et al.* 2008, Itzigsohn *et al.* 1993, Sarig *et al.* 1986, Star *et al.* 2011). Co-inoculation of *Azospirillum* and *Rhizobium* or *Bradyrhizobium* conduces to earlier nodulation or enhancement of nodule number and nodule weight (Burdman *et al.* 1997, Groppa *et al.* 1998, Molla *et al.* 2001). *A. brasilense* also allows a more persistent exudation of flavonoids by bean roots under salt stress (Dardanelli *et al.* 2008). However, some combinations do not appear more effective than single inoculations, and other seem even less effective (Cassán *et al.* 2009, Felici *et al.* 2008).

The formulation of *Azospirillum* inoculants and the choice of the carrier also determine the success of agronomic applications (Bashan 1998). Organic or not, defined or not, the optimal carrier should be economic and easy to use (Bashan *et al.* 2004); the carrier must ensure microorganisms survival and plant root colonization in field.

To conclude, in addition to technical issues, a few key determinants must be taken into account to optimize future uses of *Azospirillum* containing inoculants. Interaction specificity in *Azospirillum*-plant associative symbiosis is still an open issue, and the existence of preferential interactions between a particular bacterial strain and a particular plant genotype is overlooked. Because one strain cannot be successful worldwide, the consideration of microbial consortia containing both a large variety of *Azospirillum* strains and a wide range of beneficial microorganisms appears to be the most efficient strategy. The agronomical interest of *Azospirillum* is not limited to cereal growth promotion, as biocontrol and *Rhizobium*-"helping" are promising properties. However, a rational balance between the levels of chemical and biological fertilizers should enhance fertilization efficiency.

Concluding Remarks

There is no definite agreement on exactly how *Azospirillum* achieves plant growth promotion; it is very likely that *Azospirillum* effect results from a combination of different mechanims (Bashan and de-Bashan 2010), and that this combination differs from one strain to another. Numerous studies have been dedicated to nitrogen fixation and production of

phytohormones, the two hallmarks of this genus. Biocontrol abilities and induction of resistance by *Azospirillum* deserve to be further investigated; however, plant growth promotion is supposed to enhance the competitiveness of *Azospirillum* in the rhizosphere and the robustness of the plant, suggesting that biocontrol and stimulation of growth are not independent from each other.

The potential of *Azospirillum* for agronomic uses is undeniable, especially to replace chemical agents employed for growth promotion and control of pathogens. Thus, it is essential to better understand the mechanisms that constrain the accomplishment of an effective associative symbiosis between *Azospirillum* and the plants. The consideration of potential host specificity, underlined by the bacterial and plant genotype-specific effects observed, appears essential. For this purpose, studies of bacterial and plant genomes, transcriptomes and proteomes should provide important information on key genetic determinants involved in associative symbiosis, and could bring important clues on evolution dynamics and on events that led to specific adaptation.

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Annexe II

Genome sequence of *Azospirillum brasilense* CBG497 and comparative analyses of *Azospirillum* core and accessory genomes provide insight into niche adaptation.

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Contribution :

- Phylogénie des souches d'*Azospirillum*
- Analyse comparative des gènes impliqués dans les systèmes de sécrétion

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Article

Genome Sequence of *Azospirillum brasilense* CBG497 and Comparative Analyses of *Azospirillum* Core and Accessory Genomes provide Insight into Niche Adaptation

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Abstract: Bacteria of the genus *Azospirillum* colonize roots of important cereals and grasses, and promote plant growth by several mechanisms, notably phytohormone synthesis. The genomes of several *Azospirillum* strains belonging to different species, isolated from various host plants and locations, were recently sequenced and published. In this study, an additional genome of an *A. brasilense* strain, isolated from maize grown on an alkaline soil in the northeast of Mexico, strain CBG497, was obtained. Comparative genomic analyses were performed on this new genome and three other genomes (*A. brasilense* Sp245, *A. lipoferum* 4B and *Azospirillum* sp. B510). The *Azospirillum* core

genome was established and consists of 2,328 proteins, representing between 30% to 38% of the total encoded proteins within a genome. It is mainly chromosomally-encoded and contains 74% of genes of ancestral origin shared with some aquatic relatives. The non-ancestral part of the core genome is enriched in genes involved in signal transduction, in transport and in metabolism of carbohydrates and amino-acids, and in surface properties features linked to adaptation in fluctuating environments, such as soil and rhizosphere. Many genes involved in colonization of plant roots, plant-growth promotion (such as those involved in phytohormone biosynthesis), and properties involved in rhizosphere adaptation (such as catabolism of phenolic compounds, uptake of iron) are restricted to a particular strain and/or species, strongly suggesting niche-specific adaptation.

Keywords: *Azospirillum*; core genome; chromid; horizontal gene transfer; orthologous groups; rhizosphere

1. Introduction

Bacteria of the genus *Azospirillum* colonize roots of important cereals and grasses, and promote plant growth by several mechanisms, notably phytohormone synthesis [1,2]. Besides their potential as biofertilizer, some strains can also benefit plant health through biological control of phytoparasitic plants [3] or bacterial pathogens [4], or by inducing disease resistance [5]. In addition, *Azospirillum* may have applications in bioremediation of wastewater as it can increase the growth of microalgae commonly used in this process, such as *Chlorella* [6]. In order to rationalize the use of *Azospirillum*, genetics studies on amenable strains have mainly focused on genes involved in nitrogen fixation, auxin synthesis and on properties linked to survival in the rhizosphere [1,2,7]; knowledge of the gene repertoire of several strains may provide new insights into the *Azospirillum*-plant association.

Pioneer studies have shown that genomes of *Azospirillum* are constituted of multiple replicons and their size varies among species from 4.8 Mbp to 9.7 Mpb [8,9]. Recently, the genomes of four strains belonging to different species, isolated from various host plants and locations, were sequenced and published. The genome of *Azospirillum* sp. B510, a strain isolated from disinfected rice stems in Japan, has a size of 7.6 Mbp and consists of a single chromosome (3.31 Mbp) and six plasmids [10]. The genome of *Azospirillum amazonense* Y2, a strain isolated from the gramineous *Hyparrhenia rufa* in Brazil, was reported earlier to be constituted of four replicons of 2.7 Mbp, 2.2 Mbp, 1.7 Mbp and 0.75 Mbp [9] and its draft sequence composed of 1,617 contigs was published recently [11]. Genomes of *Azospirillum lipoferum* 4B, a strain isolated from rice in France, and *Azospirillum brasilense* Sp245, a strain isolated from wheat in Brazil, both carry seven replicons and display genome sizes of respectively 6.8 and 7.5 Mbp [12]. Whereas the largest replicon has all the features of a bacterial chromosome, several replicons could be defined as chromids [13] and some strains of *Azospirillum* appear to possess the largest number of chromids among all prokaryotic genomes sequenced to date [12]. Moreover, very little synteny was found between replicons of *Azospirillum* strains and more genomic rearrangements could be pinpointed in *Azospirillum* genomes compared to rhizobial genomes known for their genome plasticity [12]. This extraordinary genome plasticity was previously described

experimentally in *Azospirillum*: indeed, the appearance of phenotypic variants was correlated with plasmid loss or reorganization [14] and the presence of bacteriophages was evidenced [15].

Interestingly, among the family *Rhodospirillaceae*, members of the genus *Azospirillum* have the particularity of being terrestrial and plant-associated whereas nearly all known representatives live in aquatic habitats. By using a robust scheme for detecting ancestral and horizontally transferred genes in *Azospirillum*, it was shown that nearly half of the genes whose origins could be resolved, appear to be horizontally transferred from soil and plant-associated bacteria; not surprisingly the majority of genes encoding functions critical for survival in the rhizosphere and association with plants are among those acquired by horizontal gene transfer (HGT) [12]. Moreover, separation of *Azospirillum* from their close aquatic relatives approximately coincided with the emergence of vascular plants on land [12].

In the present study, the genome sequence of another member of this genus, *A. brasilense* CBG497, a strain isolated from maize grown on an alkaline soil (pH 8) in the northeast of Mexico, was first obtained. The choice was made on this strain as it is able to stimulate maize biomass yield under greenhouse conditions [16], and was recently developed as a commercial biofertilizer [17]. Then, comparative genomics analyses were performed on the four available whole genomes in order to define the *Azospirillum* core genome. The following questions were addressed: Does the core genome contain mainly genes of ancestral origin shared with aquatic relatives? Does the core genome contain genes putatively involved in rhizosphere adaptation and interaction with plants? Which functions are specific to a strain or to a species?

2. Results and Discussion

2.1. Genomic Features of Azospirillum *Genomes*

When *A. brasilense* CBG497 was subjected to replicon analysis by the plasmid Eckhardt method, five plasmids could be evidenced [18]. Pulse-field gel electrophoresis analysis confirmed the presence of five plasmids, with estimated sizes of 1.8 Mbp, 0.73Mbp, 0.65 Mbp, 0.60 Mbp and 0.15 Mbp (data not shown). The whole genome sequence of *A. brasilense* CBG497 was obtained by the 454 pyrosequencing technology and after assembly a total of 156 contigs was obtained and clustered into six scaffolds corresponding to the six expected replicons. It comprises a chromosome of 2.9 Mbp and plasmids of 1.6 Mbp, 0.731 Mbp, 0.488 Mbp, 0.606 Mbp and 0.149 Mbp that correspond respectively to p1, p2, p3, p4 and p6 of *A. brasilense* Sp245. Thus, the same nomenclature as the one used for *A. brasilense* Sp245 was applied to designate these plasmids. According to PFGE data, only the sequence of p3 seems incomplete (estimated size of 650 kb *versus* a sequenced size of 488 kb); consequently, genome coverage is estimated at 97%–98%.

General genomic features of *A. brasilense* CBG497 and of the three other *Azospirillum* strains used in this study are presented in Table 1. Genome size ranges from 6.5 Mbp (*A. brasilense* CBG497) to 7.6 Mbp (*Azospirillum* sp. B510). All strains are composed of seven replicons except *A. brasilense* CBG497 which contains only six. For all strains, only the biggest replicon has a typical chromosomal OriC replication origin whereas all the other replicons have repABC/parAB plasmid-type replication systems. Some of the latters have been classified as chromids [12,13]; p1, p2 and p4 of *A. brasilense* CBG497 also fulfill the chromid criteria as they contain respectively 40, 6 and 9 of the essential core

genes that are found on the chromosome in other species [13,18]. The smallest replicon, p6, is a typical plasmid for all strains studied and displays the lowest of the averaged GC content, which suggests an external origin by HGT; such an observation was previously made for *Rhizobium etli* and *Rhizobium leguminosarum* [19].

Chromids and plasmids comprise the largest proportion of the total genome, with 55.2% for *A. brasilense* CBG497, 56.4% for *A. lipoferum* 4B and *Azospirillum* sp. B510, and 59.8% for *A. brasilense* Sp245. So, in addition to possessing the largest number of chromids among all prokaryotic genomes sequenced to date, *Azospirillum* has the biggest proportion of its genome on non-chromosomal replicons.

Table 1. Genomic features of *Azospirillum* strains used in this study.

Strain and features	Chromosome	p1	p2	p3	p4	p5	p6	TOTAL
***A. brasilense* CBG497**								
size of replicon[§]	2,900,071	**1,598,241**	**731,389**	488,405	**606,415**		148,687	6,473,208
G+C content	68.4	68.8	68.8	66.05	69.3	Absent	67.1	68.4
number of ORFs	2895	1430	643	512	583		122	6185
rRNA	nk	1	1	nk	nk		0	nk
tRNA	42	16	1	2	6		0	67
***A. brasilense* Sp245**								
size of replicon	3,023,440	**1,766,028**	**912,449**	778,798	**690,334**	191,828	167,364	7,530,241
G+C content	68.6	68.6	68.3	68.2	69.0	66.7	66.8	68.5
number of ORFs	3309	1812	922	824	691	163	125	7846
rRNA	2	3	2	0	1	0	0	8
tRNA	44	25	2	0	9	0	1	81
***A. lipoferum* 4B**								
size of replicon	2,988,332	**1,040,425**	**750,123**	**648,491**	**645,253**	**478,032**	295,744	6,846,400
G+C content	67.6	67.6	67.6	67.8	68.3	67.7	67.1	67.7
number of ORFs	2904	883	640	555	599	415	237	6233
rRNA	2	3*	2	1	0	1[†]	0	9
tRNA	46	12	5	2	6	8	0	79
***Azospirillum sp.* B510**								
size of replicon	3,311,395	**1,455,109**	**723,779**	**681,723**	**628,837**	**537,299**	261,596	7,599,738
G+C content	67.8	67.6	67.5	67.4	68.0	67.5	65.9	67.6
number of ORFs[#]	3287	1263	693	589	598	464	232	7126
rRNA	2	4	1	1	0	1	0	9
tRNA	45	14	2	3	6	9	0	79

[§] When the size is indicated in bold, the chromid definition applies to the corresponding replicon, *i.e.* plasmid-type maintenance replication systems, presence of essential genes and a nucleotide composition close to that of the chromosome [12,13]; [¶] The third criteria of chromid definition (nucleotide composition close to that of the chromosome) does not apply for these two replicons; *The 5S rRNA is missing from one of the operons; [†] The 23S rRNA is absent; [#] The number of ORFs corresponds to the one established after the sequence was imported and annotated into the MaGe platform [78]; nk: not known.

2.2. Genomic Relatedness between Azospirillum *Strains*

Before undertaking comparative genomic analysis, the relatedness between *Azospirillum* sp. B510 and the other *Azospirillum* strains used in this study was clarified. *Azospirillum* sp. B510 was previously shown to be related to the species *A. lipoferum* to which it was originally affiliated [20]. Subsequent studies revealed that it was closer to the species *A. oryzae* than to the species *A. lipoferum* [10]. Since new species of *Azospirillum* have recently been described, a 16S rRNA phylogenetic tree was constructed, and this confirmed that strain B510 was more closely related to the species *A. oryzae* and *A. zeae* (Supplementary Figure S1). Recently, the average nucleotide identity was determined between *A. lipoferum* 4B and *Azospirillum* sp. B510, and was found to be 91% [12] supporting the fact that these two strains belong to different species [21].

A first comparative analysis was undertaken in order to classify proteins into families and hence to evaluate the genomic relatedness among *Azospirillum* strains in terms of protein coding content. As the draft sequence of *A. amazonense* Y2 is composed of 1,617 contigs with only 3,319 predicted CDS, far fewer than what is expected for its 7.3 Mbp genome-size, it was excluded for the comparative analysis. The predicted proteins of the four remaining *Azospirillum* genome sequences (a total of 27,400 proteins) were clustered using the MCL algorithm [22]. About 47% of the protein families identified (2,600 out of 5,575) are shared by the four strains (Figure 1); the two *A. brasilense* strains share 74% of the protein families (4,136 out of 5,575) whereas the *A. lipoferum* and *Azospirillum* sp. pair share 66% of the protein families (3,667 out of 5,575). Other combinations share between 51.2% and 54.6% of the protein families; these results are in accordance with *A. lipoferum* 4B and *Azospirillum* sp. B510 belonging to different species but to species more closely related than the *A. brasilense* and *A. lipoferum* species. A number of protein families are exclusive to individual genomes (Figure 1). As for proteins that do not appear in any protein families, their number is quite variable from one strain to another (949 for *A. brasilense* CBG497; 2,311 for *A. brasilense* Sp245; 1,203 for *A. lipoferum* 4B; 1,768 for *Azospirillum* sp. B510) and not surprisingly is in direct correlation with genome size. Repartition of these unique proteins among replicons shows that the biggest proportion (>60%) is located outside the chromosome, and that the repartition is variable from one strain to another (Figure 2). For each genome, the array of unique proteins contain approximately 20% of ancestral proteins, 30 to 43% of proteins previously classified as horizontally acquired and the rest being classified as unassigned [12]; this last category consists of proteins having no orthologues in the nr database of Genbank. Some of the unique proteins with assigned functions will be discussed in subsequent sections.

2.3. The Azospirillum *Core Genome*

The most probable set of orthologous proteins shared by the four *Azospirillum* strains and by the phylogenetically related *Rhodospirillum centenum* SW strain was identified by a reciprocal best blast hit criterion. *Rhodospirillum rubrum* was not included in the analysis as this strain is more related to the genus *Magnetospirillum* than to *Azospirillum* (data not shown). A total of 1,151 proteins is shared by these five strains (Supplementary Table S1); this minimal gene set can be considered as the "ancestral" core genome (designated ANC-core) as it contains nearly exclusively (95%) proteins previously classified as ancestral using the scheme developed previously [12]. As expected, the

ANC-core is largely encoded by the chromosome and by p1 (from 85% to 90%) in the four *Azospirillum* strains (Figure 3A).

Figure 1. Venn diagram showing the distribution of protein families in the genomes of *A. brasilense* CBG497, *A. brasilense* Sp245, *A. lipoferum* 4B, *Azospirillum* sp. B510. Numbers in black indicate the number of protein families; numbers in parenthesis and highlighted in grey refer to the number of unique proteins in each genome that do not fall in any family.

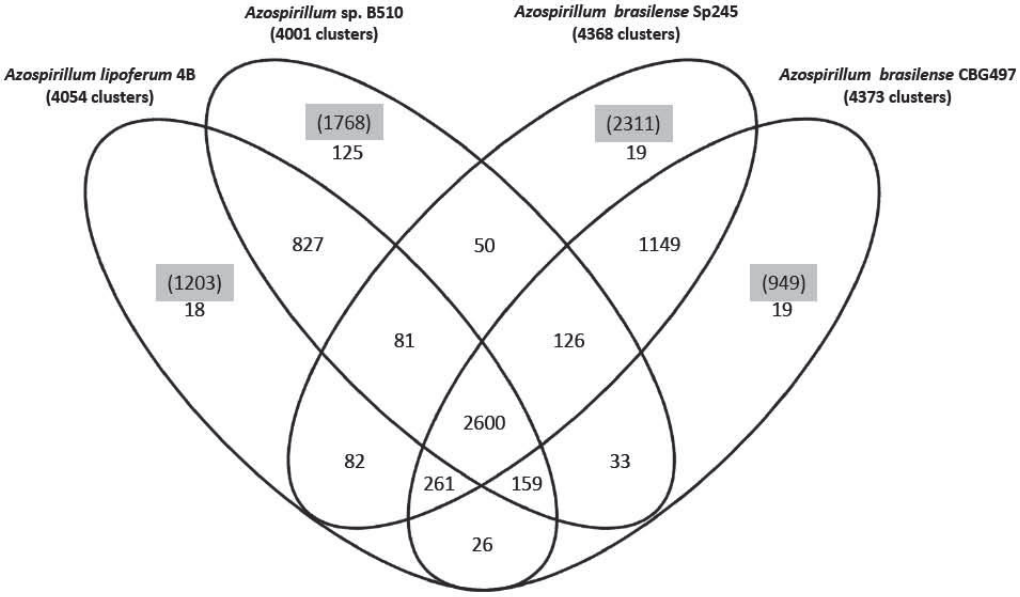


Figure 2. Repartition among replicons of *Azospirillum* unique proteins. Color legend: Grey (chromosome), burgundy (p1), yellow (p2), light blue (p3), purple (p4), orange (p5), dark blue (p6).

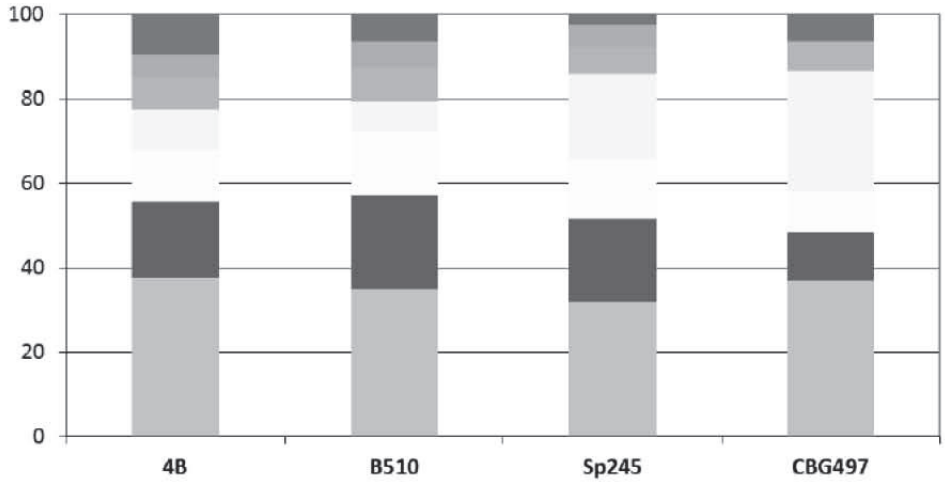
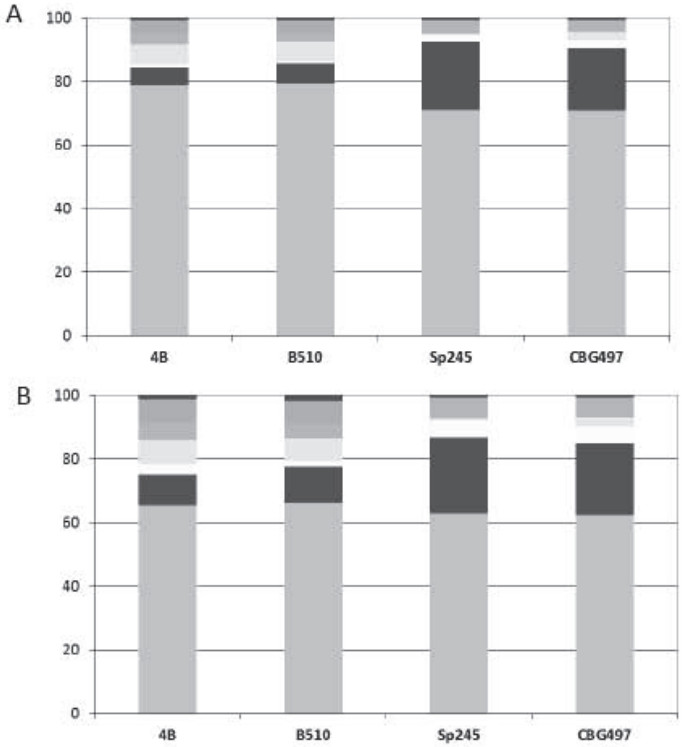


Figure 3. Repartition of orthologous groups among replicons for each *Azospirillum* strain. (A) Repartition of the 1,151 *Azospirillum-Rhodospirillum* orthologous groups (ANC-core). (B) Repartition of the 2,328 *Azospirillum* orthologous groups (AZO-core). Color legend: Grey (chromosome), burgundy (p1), yellow (p2), light blue (p3), purple (p4), orange (p5), dark blue (p6).



The same procedure was applied to identify the *Azospirillum* core genome (designated AZO-core) *i.e.*, the set of orthologous proteins shared by the four *Azospirillum* strains. A total of 2,328 proteins (including the 1,151 of the ANC-core) is shared by the four strains, representing between 30% to 38% of the total encoded proteins within a genome (Supplementary Table S1). A similar study undertaken on four other *Rhodospirillaceae* belonging to the *Magnetospirillum* genus estimated the magnetobacterial core genome at about 891 genes, which represents 18 to 24% of the total proteins encoded by those genomes [23]. Three different species of *Streptococcus* were shown to share around half of their genes [24].

The AZO-core set is also dominated by proteins of ancestral origin (74%) but contains more than a fifth (22%) of proteins encoded by horizontally acquired genes and a small proportion of proteins encoded by genes whose origin could not be resolved (4%). The repartition among replicons shows that AZO-core is mainly chromosomally-encoded (from 62% to 65% according to the strain considered) (Figure 3B). The non-chromosomal proportion of AZO-core is unevenly distributed among strains. There is a strong dominance of p1 in the *A. brasilense* strains (p1 > p4 > p2), which might be attributable to the size of this replicon; genes of the AZO-core that are p1-encoded in *A. brasilense* strains are found on the chromosome in the two other strains. For *A. lipoferum* and

Azospirillum sp. strains, the non-chromosomal proportion of AZO-core is mainly found on p1, p3 and p5 (Figure 3B); orthologues of those p3- and p5-encoded genes are scattered on the different replicons bearing AZO-core genes in *A. brasilense* (*i.e.*, chromosome, p1, p2 and p4) (data not shown). So, it appears that p3, p5 (absent in CBG497) and p6 are accessory replicons for *A. brasilense*, which is in accordance with their plasmid (versus chromid) status. For *A. lipoferum* 4B and *Azospirillum* sp. B510, the unique defined plasmid p6, appears to be a dispensable replicon. The p2 chromid of those two strains bears very few AZO-core genes, which is in accordance with the very small number of house-keeping genes previously identified on this replicon [12] and with the observation of p2 loss at high frequency in *A. lipoferum* 4B [14].

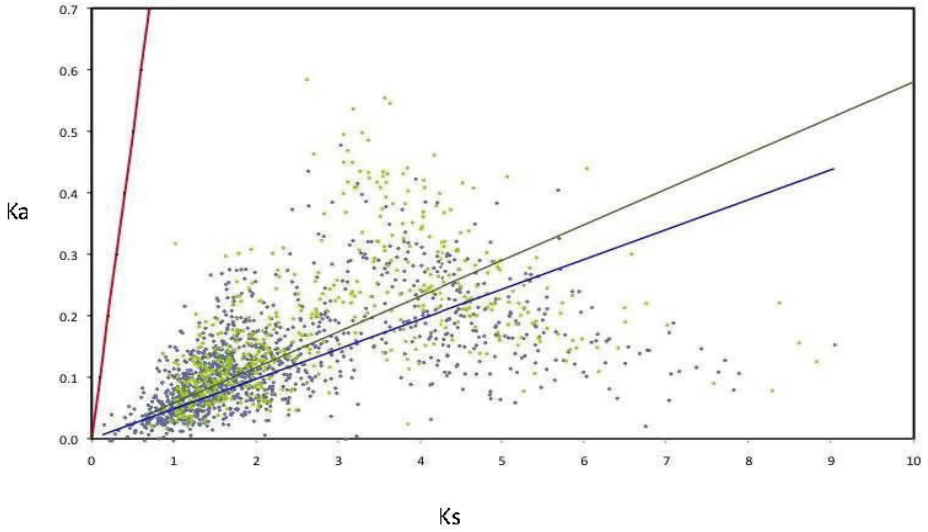
As expected, the COGs that are overrepresented in the ANC-core are mainly those involved in "house-keeping" functions: COGs J (Translation, ribosomal structure and biogenesis), C (Energy production and conversion), O (Post-translational modifications, protein turnover, chaperones), M (Cell wall / membrane / envelope biogenesis), E (Amino acid transport and metabolism) and H (Coenzyme transport and metabolism) (Figure 4).

A particular focus on genes found in the AZO-core that are absent in the ANC-core shows that *Azospirillum* is more adapted to life in fluctuating environments; indeed the AZO-core contains many additional genes involved in signal transduction (COG T, such as two-component histidine kinases, diguanylate cyclases, methyl-accepting chemotaxis receptors) and regulation of transcription (COG K); around 8% of the additional genes encode regulators of two-component systems, transcriptional regulators (mainly LysR, GntR and LuxR-FixJ families) and sigma factors (Figure 4). Adaptation to the rhizosphere is also illustrated by the enrichment of COG G and more importantly COG E, corresponding respectively to "carbohydrate transport and metabolism" and to "amino acid transport and metabolism" (this latter category also includes metabolism of organic acids, compounds that are abundant in the rhizosphere). *Azospirillum* has acquired numerous transport systems (representing 18% of the genes that are specific to the AZO-core); those transporters, mainly ABC transporters (scattered through COGs E, G, and I), can serve to internalize the wide diversity of organic and mineral compounds present in the rhizosphere (notably organic compounds exuded by plant roots) or to expel putative plant toxic compounds via MDR efflux pumps. Genes likely involved in bacterial surface properties are also enriched (COG M), such as those involved in the processing of complex sugars (LPS, EPS), increasing the ability of *Azospirillum* to attach to roots. Genes involved in direct plant-growth promotion do not belong to the AZO-core and seem to have been gained specifically after speciation events or by individual strains (see below). One exception is the PQQ operon, allowing the synthesis of the cofactor pyrroloquinoline quinone, a compound displaying plant growth-promoting properties [25].

The rates of evolution were evaluated for the components of the AZO-core; in order to perform this, the rates of nucleotide substitution per synonymous (Ks) and non-synonymous (Ka) were calculated for a subset of 1,807 AZO-core genes (for those being chromosomal in all strains and those being non-chromosomal in all strains). All the orthologous groups are under negative selection (Figure 5). Nevertheless many non-chromosomal genes show higher Ka and Ks values than the chromosomal genes, as illustrated by the slopes of the regression lines, suggesting that negative selection is less constrained for genes outside the chromosome. Such an observation was previously made with the *Rhizobium* core genome [19].

Figure 4. COGs functional classification of the *Azospirillum* orthologous groups. Bars indicate the numbers of orthologous groups for each COG retrieved from the MaGe platform for the *A. lipoferum* 4B orthologues. For each bar, the grey part represents the number of orthologous groups found in the ancestral (*Azospirillum-Rhodospirillum)* orthologous groups, the green part represents the number of orthologous groups that are unique to the *Azospirillum* genus. COG: J, Translation, ribosomal structure and biogenesis; K, Transcription; L, Replication, recombination and repair; D, Cell cycle control; V, Defence mechanisms; T, Signal transduction mechanisms; M, Cell wall, membrane envelope biogenesis; N, Cell motility; U, Intracellular trafficking and secretion; O, Postranslational modification and chaperones; C, Energy production and conversion; E, Amino acid transport and metabolism; F, Nucleotide transport and metabolism; G, Carbohydrate transport and metabolism; H, Lipid transport and metabolism; I, Coenzyme transport and metabolism; P, inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; R, General function prediction; and S, Function unknown.

Figure 5. Rates of synonymous (Ks) and non-synonymous substitutions (Ka) in orthologous genes of *Azospirillum*. Linear regressions for chromosomal orthologous genes (blue line and diamonds) and non-chromosomal orthologous genes (green line and diamonds) are indicated. As neutrality (red line) assumes equal nucleotide substitution rates per synonymous and non-synonymous sites, points under the neutrality line indicate negative selection. Strong selective constraints are acting on chromosomal genes (R2 = 0.127) but are slightly less intense for non-chromosomal genes (R2 = 0.092) as can be seen by the dispersion of the green diamonds.



2.4. Strain-specific Genes Involved in the Colonisation of Plant Roots

The focus was then put on genes with assigned functions that are restricted to a unique strain or to a subgroup of *Azospirillum* strains. A part of the corresponding genes falls into unique protein families described earlier (see section 2.2 and Figure 1) and have mainly been acquired by horizontal gene transfer. Their relevance to interaction with plants and adaptation to the rhizosphere is discussed.

Chemotaxis and motility are primordial for the initiation of root colonization in a wide range of rhizobacteria. The chemotaxis system integrates environmental signals into an appropriate bacterial response by using a dedicated signal transduction pathway. Whereas the AZO-core contains genes implicated in flagellum biosynthesis and genes belonging to the four common chemotaxis operons, specific genes confirm the presence of one and two additional operons respectively in *A. lipoferum* 4B and in *Azospirillum* sp. B510 [12]. Consequently, those two strains, and notably *Azospirillum* sp. B510, contain a significant number of unique genes encoding methyl-accepting proteins, able to detect various physicochemical cues and to relay information to the flagellar motors via a signal transduction cascade.

Interactions between plant and bacterial polysaccharides are thought to mediate bacterial aggregation and attachment processes [26,27]. The AZO-core contains genes whose involvement in biosynthesis of exopolysaccharide (EPS) and lipopolysaccharide (LPS) was demonstrated, such as *noeL*, *noeJ* and *rmlD* [7,28]; however, the presence of several unique genes suggests that EPS and LPS

components might differ from one *Azospirillum* strain to another, a feature previously reported for LPS [7]. Only *A. brasilense* strains possess additional clusters of ancestral genes involved in EPS biosynthesis and/or transport (such as AZOBR_p310279, AZOBR_p330029, AZOBR_p60088, AZCBG_p60114, AZCBG_230032). Besides, *A. brasilense* genomes contain an additional gene involved in LPS biosynthesis and acquired by HGT (AZCBG_p190020; AZOBR_p210177). These genes might be relevant for the adaptation of azospirilla strains to their environment [29].

Azospirillum strains have gained different root-adhesion mechanisms. Indeed, TAD pili are exclusive to the *A. brasilense* species; these pili play an essential role in biofilm formation, colonization and pathogenesis in various genera [30] and their role in biofilm formation was recently assessed in *A. brasilense* Sp245 [12]. Cellulose synthesis is another mechanism by which bacteria can tightly bind to the roots [31]. Gene(s) involved in cellulose synthesis and acquired by HGT are found exclusively in *A. lipoferum* 4B and *Azospirillum* sp. B510.

Cellulases and hemicellulases likely contribute to endophytic plant colonization, a property described for *A. brasilense* Sp245 and *Azospirillum* sp. B510 [20,32]. *Azospirillum* genomes were previously shown to encode a substantial number of glycosyl hydrolases (from 26 to 34), some of them likely capable of degrading plant cell wall [12]. However, some CAZy families are restricted to a species; genomes of *A. lipoferum* 4B and *Azospirillum* sp. B510 encode cellulases belonging to the GH8 and GH16 (licheninase) families whereas *A. brasilense* strains possess an endoglucanase of the GH12 family (AZOBR_p440082 and AZCBG_p490023). Both *A. brasilense* strains possess unique glycosyl hydrolase-encoding genes with no orthologues in any other *Azospirillum* genomes.

2.5. Strain-Specific Genes Involved in Plant Growth Promotion

The contribution of nitrogen fixation to plant growth promotion by *Azospirillum* is controversial; however, greenhouse and field inoculation experiments significantly reduced the required dozes of nitrogen fertilization [1]. In addition to the nitrogen fixation cluster that has been inherited vertically, *A. brasilense* Sp245 genome harbours a second cluster for nitrogen fixation (AZOBR_p350011 to AZOBR_p350024). This cluster encodes a vanadium nitrogenase and may have been horizontally transferred from *Rhodopseudomonas palustris*; an integrase lies just downstream of this operon (AZOBR_p350032). As the genome of *A. brasilense* CBG497 is not closed (part of the p3 is missing), the occurrence of this operon was searched by PCR: amplification with primers for *vnfX* (AZOBR_p350013), *vnfD* (AZOBR_p350020) and *vnfH* (AZOBR_p350022) was negative, suggesting that this operon has been acquired recently by *A. brasilense* Sp245.

Phosphate solubilization represents another important trait for enhancement of plant nutrition [33]. Interestingly, *A. lipoferum* 4B and *Azospirillum sp.* B510 may have acquired the ability to solubilize inorganic phosphates through the secretion of gluconic acid. Oxidation of glucose into gluconic acid takes place in the periplasm and is assumed by a PQQ-dependent glucose dehydrogenase (AZOLI_p50302 / AZL_e01560) [34]. No orthologue was found in the *A. brasilense* genomes; such a property which has been scarcely reported in the *Azospirillum* genus [35] deserves to be investigated.

Plant growth promotion by *Azospirillum* is thought to occur essentially through modulation of the plant hormonal balance via the synthesis of phytohormones, notably the phytohormone indole-3-acetic acid (IAA), or via the degradation of phytohormones or precursors of phytohormones, such as

1-aminocyclopropane-1-carboxylic acid (ACC) [36]. *A. brasilense* Sp245 is well-known to produce IAA from tryptophan (Trp) through the indole-3-pyruvate (IPyA) biosynthetic pathway [37–39]. One key enzyme of this pathway, encoded by *ppdC*/*ipdC*, is the indole-3-pyruvate decarboxylase, which mediates conversion of indole-3-pyruvate into indole-3-acetaldehyde (second step of the pathway). This gene is present in the genome of the two *A. brasilense* strains (AZOBR_40354 and AZCBG_190067), but absent from genomes of *A. lipoferum* 4B and *Azospirillum* sp. B510. The first and third steps of the IPyA pathway correspond respectively to the conversion of tryptophan into IPyA and of indole-3-acetaldehyde into IAA. These steps are catalyzed by enzymes, respectively aromatic aminotransferases and NAD-dependent aldehyde dehydrogenase, which are common and non-specific enzymes. Recently, the contribution of *hisC1*, which encodes an aromatic amino acid aminotransferase-1 (AAT1), to IAA production was evidenced in *A. brasilense* Sp7 [40]. Homologues sharing identity levels higher than 75% with AAT1 from Sp7 were found in all four *Azospirillum* genomes (AZOLI_1579, AZCBG_330158, AZOBR_120044, AZL_012940).

In Sp245, it was shown that an *ipdC* knockout mutant still produced 10% of the wild-type IAA production level [39], indicating that other metabolic pathways contribute, even though to a lesser extent, to IAA production. Indeed, a large set of genetic and biochemical studies strongly suggest that *A. brasilense* might possess a triptamine (TAM) and an indole-3-acetonitrile (IAN) pathway [41,42]. The IAN pathway corresponds to the conversion of Trp into indole-3-acetaldoxime (IAox), of IAox into IAN and then of IAN into IAA. In *Arabidopsis thaliana,* two cytochrome P450 enzymes (CYP79B2 and CYT79B3) catalyze the formation of IAox from Trp, and two nitrilase genes *NIT1* and *NIT2* have been shown to contribute to IAA biosynthesis *in vivo* [41,43]. P-blast search performed on *Azospirillum* genomes with the two nitrilase protein sequences from *A. thaliana* revealed a putative nitrilase in *A. brasilense* Sp245 (AZOBR_p350044, respectively 48.9% and 49.84% identity with NIT1 and NIT2) and in *Azospirillum sp.* B510 (AZL_020600, respectively 36.09% and 34.78% identity); those genes were previously classified as HGT [12].

The indole-3-acetamide pathway (IAM) involves the decarboxylation of Trp into IAM by a Trp monooxygenase (*iaaM*), and the hydrolysis of IAM into IAA by an indole acetamide hydrolase (*iaaH*). The existence of this pathway was suggested in *Azospirillum sp.* B510, with candidates for *iaaM* and *iaaH* represented respectively by AZL_b03560 and AZL_b03580 [10], two genes that are unique to the B510 genome. However, AZL_b03560 appears rather encoding a triptamine oxidase that might be involved in the conversion of TAM into indole-3-acetaldehyde. In B510, it seems that IAM is produced rather from the conversion of IAN to IAM by a nitrile hydratase rather than directly from Trp by a Trp monooxygenase. Consistently, no homologues of *iaaM* from *Agrobacterium tumefaciens*, *Dickeya dadantii* or *Pseudomonas syringae* pv. *syringae* were found in any *Azospirillum* genomes. Interestingly, eight clustered genes unique to the B510 genome and previously classified as HGT encode putative nitrile hydratases (AZL_a09780, AZL_a09790, AZL_a09810, AZL_a09820, AZL_a09830, AZL_a09840, AZL_a09850, AZL_a09860) and are located near a transposase and a tRNA. Future studies are required to verify if all those genetic determinants are implicated in IAA biosynthesis in those strains, since HPLC analyses revealed the ability to produce IAA in the presence of Trp for *A. brasilense* Sp245 and CBG497 but this production was negligible for *A. lipoferum* 4B and *Azospirillum sp.* B510 (our unpublished results).

Besides the ability to produce IAA, it was investigated whether *Azospirillum* strains are able to catabolize this phytohormone. IAA catabolism has been characterized in some rhizobacteria such as *Pseudomonas putida*, and relies on the presence of the *iac* locus (for IAA catabolism) constituted of 10 genes with coding similarity to enzymes acting on indole or amidated aromatics and to proteins with regulatory or unknown function [44,45]. Homologs of the *iac* genes are present in *A. lipoferum* 4B (AZOLI_p10981 to AZOLI_p10991) and *Azospirillum sp.* B510 (AZL_a08890 to AZL_a08810). The IAA catabolism phenotype was investigated and a slight growth on 5 mM IAA as sole carbon and nitrogen source was observed in the two strains while no growth was observed in the two *A. brasilense* strains (data not shown). Thus, it appears that, unlike *A. brasilense* strains, *A. lipoferum* 4B and *Azospirillum sp.* B510 may not be able to produce IAA but can metabolize such a substrate.

The deamination of ACC is another key activity involved in the modulation of the plant hormonal balance by rhizobacteria. ACC is the immediate precursor of plant ethylene, and its deamination leads to a decrease of ethylene production in plants. Because ethylene inhibits root growth and may be produced in too large amounts during plant stress response, bacterial ACC deamination can enhance both root system development and plant stress tolerance [46]. The ACC deaminase activity is encoded by *acdS* that is widely distributed in Proteobacteria; among the *Azospirillum* genus, this gene is mostly harboured by strains of the *A. lipoferum* species and has been acquired by HGT [12,47,48]. Accordingly, *acdS* and *acdR* (encoding a *lrp*-like transcriptional activator of *acdS*) are absent from the *A. brasilense* Sp245 and CB497 genomes, whereas they lie on the second largest chromid in *A. lipoferum* 4B (*i.e.*, AZOLI_p20559 and AZOLI_p20560) and in *Azospirillum sp.* B510 (*i.e.*, AZL_b04170 and AZL_b04180).

The plant hormonal balance might also be modulated by the degradation of salicylate into catechol via salicylate 1-monooxygenase (EC 1.14.13.1). Such an enzyme was identified in *A. lipoferum* 4B and in *A. brasilense* strains (AZOLI_p20435 / AZOBR_p480008 / AZCBG_p410058). AZOLI_p20435 displays only 36-37% identity with the *A. brasilense* orthologs and 81% to NahG of *Pseudomonas fluorescens* SBW25 whereas the *A. brasilense* orthologs display strong identity (67%–68%) with NahW of *Burkholderia xenovorans* LB400; this observation is consistent with two independent acquisitions through HGT [12]. Nevertheless only *A. lipoferum* 4B harbors the metabolic pathway for catechol degradation (see below) and thus may use salicylate as a source of energy and carbon.

2.6. Strain-Specific Catabolic Pathways Involved in Adaptation to the Rhizosphere

Examination of strain-specific genes revealed specific catabolic properties that might be relevant for adaptation to the rhizosphere. A complete ribose degradation pathway was identified in *A. lipoferum* 4B and *Azospirillum sp.* B510 involving a ribokinase (*rsbK*, AZOLI_p20179 / AZL_b03490), a deoxyribokinase/ribokinase (*deoK*, AZOLI_p20643 / AZL_b05870), and a deoxyribose mutarotase (*deoM*, AZOLI_p20642 / AZL_b05860). The catabolic pathway of myo-inositol was identified only in *Azospirillum sp.* B510 (AZL_b00950 and AZL_b01030 to AZL_b01060).

A. lipoferum 4B and *Azospirillum sp.* B510 may have the ability to degrade the organophosphonate 2-aminoethylphosphonate. Organophosphonates are quite abundant in nature, primarily as components of phosphonolipids, but also as constituents of polysaccharides, glycoproteins, glycolipids and several antibiotics. The enzymes catalyzing the first two steps, *i.e.*, 2-aminoethylphosphonate-pyruvate

transaminase (*phnW*, AZOLI_p20203 / AZL_a10490) and phosphonoacetaldehyde hydrolase (*phnX*, AZOLI_p20204 / AZL_a10480) are present only in those two genomes.

Azospirillum sp. B510 may degrade and use the aliphatic amine methylamine as a nitrogen source. Various aliphatic amines can be emitted in agricultural systems, notably methylamine (MMA), dimethylamine and trimethylamine [49]. An alternative MMA oxidative pathway different from the direct oxidation carried out by MMA dehydrogenase was fully characterized on *Methyloversatilis universalis* FAM5 [50]. This eight gene cluster composed of *mgdABCD*, *gms* and *mgsABC* is present in other methylotrophs [51,52] and in nonmethylotrophs, including *Agrobacterium tumefaciens* C58 which can grow using MMA as sole nitrogen source. The genome of *Azospirillum* sp. B510 carries this cluster (AZL_a09510 to AZL_a09580), next to *purU* and *folD* encoding respectively formyltetrahydrofolate deformylase and methylenetetrahydrofolate dehydrogenase/cyclohydrolase that are necessary for the detoxification of formaldehyde generated by this metabolic pathway [53].

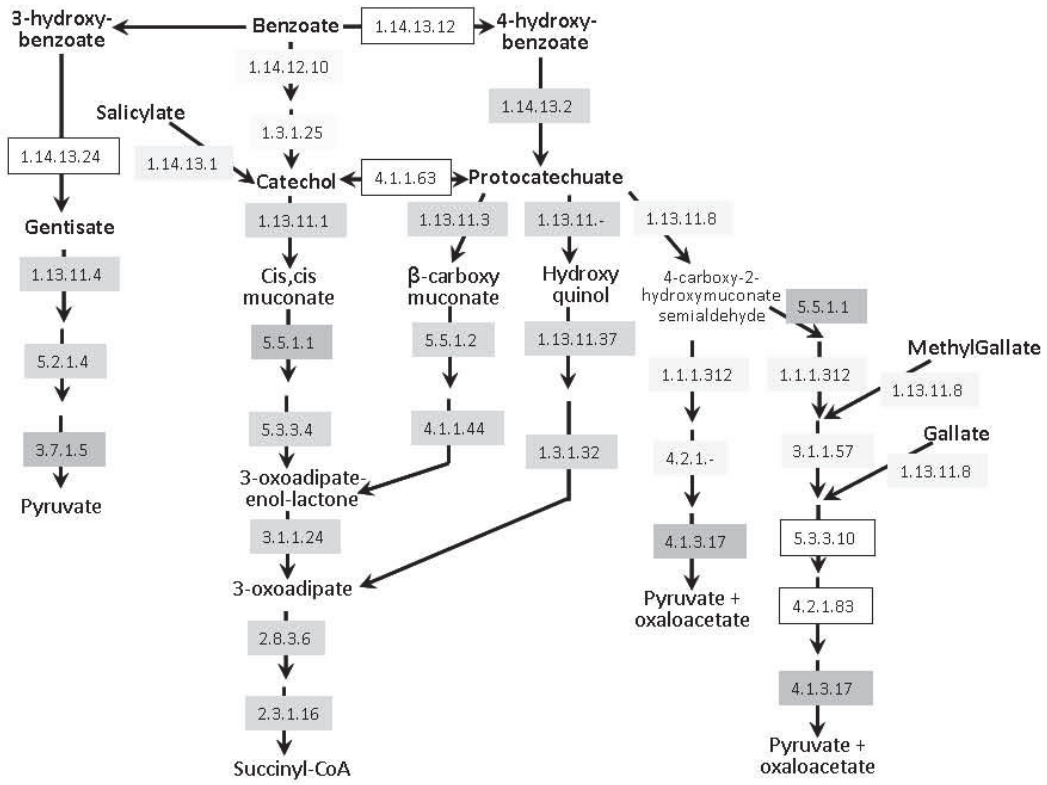
Degradation of aromatic compounds (*i.e.,* organic molecules containing one or more aromatic rings mainly produced by plants) is dominated by aerobic and anaerobic bacteria and aerobic fungi [54]. The aerobic catabolism of aromatic compounds usually involves the oxygenolytic hydroxylation of the aromatic ring, producing central dihydroxylated aromatic intermediates (e.g., catechol, protocatechuate, gentisate, homoprotocatechuate, homogentisate and hydroxyhydroquinone). These intermediates are then cleaved by different types of ring-cleavage dioxygenases, generating aliphatic compounds that funnel into the tricarboxylic acid (TCA) cycle through a small number of central pathways [55]. Degradation of a broad spectrum of aromatic natural and xenobiotic compounds relies on two central intermediates: catechol and protocatechuate. *A. lipoferum* 4B and *Azospirillum sp.* B510 can degrade these compounds via the β-ketoadipate pathway (Figure 6); they also possess enzymes allowing the conversion of benzoate and 4-hydroxybenzoate into protocatechuate. In addition, *Azospirillum sp.* B510 can convert benzoate into catechol. Both strains can also metabolise gentisate (2,5-dihydroxybenzoate). *A. brasilense* strains seem to be less versatile as only the meta-cleavage pathway of protocatechuate degradation has been identified; catabolism of gallate and methylgallate might be present but one of the key enzymes (EC 4.2.1.83) could not be identified. Experimentally, growth on protocatechuate as the sole carbon source was observed for all strains except *A. brasilense* CBG497 (data not shown). As for transporters, *A. brasilense* strains possess a protocatechuate transporter with strong identity to a *Bradyrhizobium* transporter (AZOBR_p310195 in Sp245 and a gene present on the missing part of p3 in CBG497 as revealed by PCR). A gene encoding hydroxybenzoate transporter is found adjacent to the gentisate degradation pathway in *A. lipoferum* 4B and *Azospirillum sp.* B510 (AZOLI_p20645 and AZL_a09170) and displays identity with *pcaK* of *Ralstonia*. PcaK functions as a chemoreceptor for chemotaxis towards aromatic acids [56]. Additional hydroxybenzoate transporters are encoded by the genome of *Azospirillum sp.* B510 (AZL_a07380 and AZL_b03660).

The phenylacetate catabolic pathway is the central route where catabolic pathways of many aromatic compounds converge and are directed to the TCA cycle [57]. The aerobic degradation of phenylacetate by epoxidation of CoA thioesters occurs in ~16% of all bacterial species with sequenced genomes [54]. Within the genomes of *Azospirillum*, only *A. lipoferum* 4B and *Azospirillum sp.* B510 strain harbour the complete *paa* catabolic cluster; 11 of these loci are located on the p4/d replicon (AZOLI_p40257 to AZOLI_p40267), whereas *paaX, paaY,* and *paaF* (which is duplicated) are located

onto the chromosome, thus constituting five different clusters, an organization previously reported in *Pseudomonas putida* [58]. Seven of the genes located on p4 (*paaA, paaB, paaC, paaD, paaE, paaN* and *paaJ*) have been classified as horizontally transferred whereas the others have been classified as ancestral [12], suggesting a complex evolution of this catabolic pathway. The functionality of the *paa* genes was assessed as both strains were able to grow on phenylacetic acid as the sole carbon source, whereas the *A. brasilense* strains showed no growth (Supplementary Figure S2).

Thus, *A. lipoferum* 4B and *Azospirillum sp.* B510 seem to be more versatile for aromatic compound degradation than *A. brasilense* strains; indeed, in addition to the above-mentioned pathways, several aromatic ring-hydroxylating dioxygenases could be identified. It will be interesting to determine if this versatility is related to the composition of the host plant exudates, as a result of niche-specific adaptation. This versatility could also be related to environmental conditions, such as soil type and cultural practices (like flooding for rice) that can greatly influence the microbial community.

Figure 6. Aromatic compounds catabolism in *Azospirillum*. Enzymes framed in grey are encoded by all genomes. Enzymes framed in orange are encoded exclusively by genomes of *A. lipoferum* 4B and *Azospirillum* sp. B510. Enzymes framed in yellow are exclusive to *Azospirillum* sp. B510. Enzymes highlighted in blue are specific to the *A. brasilense* strains (Sp245 and CBG497). Conversion of salicylate into catechol (green) is encoded by all genomes except that of *Azospirillum* sp. B510. Non-colored enzymes are missing in all strains. Enzyme 1.1.1.312 was previously known as 1.2.1.45. Alternative names for gentisate and protocatechuate are respectively 2,5-hydroxybenzoate and 3,4-hydroxybenzoate.



2.7. Other Strain-Specific Genes Likely Involved in Adaptation to the Rhizosphere

Azospirillum strains are not equally equipped for iron acquisition, an important component of bacterial metabolism. Discrepancies are observed at the level of siderophores biosynthesis and uptake. A 14 kb region that spans AZOLI_p20158 to AZOLI_p20165 is predicted to be involved in pyochelin biosynthesis and appeared to be exclusively present in the *A. lipoferum* 4B genome. The corresponding genes, classified as HGT [12], show protein identity levels greater than 45% with those of *Pseudomonas fluorescens* and a high degree of synteny. A cluster of genes involved in enterobactin biosynthesis was specifically found in the two *A. brasilense* strains (AZOBR_p350073 to AZOBR_p350079 / ACBG_p22001 to AZCBG_p22007). *A. brasilense* Sp245 possesses a unique cluster of eight genes predicted to be involved in siderophore transport (AZOBR_220054 to AZOBR_p220061); a gene encoding a *fecI*–type sigma factor is located directly upstream of this cluster (AZOBR_p220053) and is similar to PsbS/PvdS, a sigma-70 ECF of *Pseudomonas* (56% of identity at the protein level with PsbS of *Pseudomonas* sp. B10). PvdS was shown to regulate the transcription of pyoverdine biosynthesis genes under iron starvation in *P. aeruginosa* [59]. A hemin ABC transporter is also present in the genomes of *A. brasilense* Sp245 and *A. lipoferum* 4B. As plant colonization ability has been shown to be linked to iron acquisition systems [60], discrepancies observed among azospirilla could be relevant.

Plants are capable of producing reactive oxygen species (ROS), as a defence mechanism against both pathogenic and symbiotic bacteria [61,62]. Living organisms have built up mechanisms to protect themselves against oxidative stress, with antioxidant enzymes such as catalase and superoxide dismutase, small proteins like thioredoxin and glutaredoxin, and molecules such as glutathione. Comparative genomic analysis shows that next to common mechanisms, several enzymes involved in the oxidative stress response differ among *Azospirillum* species. For example, a gene encoding a superoxide dismutase (SodA) is found uniquely in *A. brasilense* strains (AZOBR_p440007 / AZCBG_p410047), whereas a gene encoding a catalase is present in *A. lipoferum* 4B and in *Azospirillum sp.* B510 (AZOLI_p10486 / AZL_a00280). Moreover, a bifunctional catalase-peroxidase (KatG) is only found in *A. lipoferum* 4B (AZOLI_p30178). The deduced amino acid sequence of this ORF have 80% identity with the KatG protein of *Rhizobium etli*, and is directly located downstream of the gene encoding the OxyR transcription factor. KatG plays a role in survival during stationary-phase in *R. etli*, but is not essential for nodulation and nitrogen fixation in symbiosis with *Phaseolus vulgaris* [63].

Components of a type VI secretion system (T6SS) have previously been identified in the three published genomes and classified as HGT [12]; however the T6SS components display discrepancies among strains. A region with an organization similar to that of Alpha-proteobacteria (such as *Azorhizobium*) is located on the chromosome of *A. lipoferum* 4B (AZOLI_0998 to AZOLI_1020) and *Azospirillum* sp. B510 (AZL_017990 to AZL_017770). A second region exclusive to *A. lipoferum* 4B (AZOLI_p30482 to AZOLI_p30489) displays similarities with T6SS of *Bradyrhizobium japonicum* USDA110. A third region unrelated to the previous ones lies on the biggest chromid of the two *A. brasilense* strains and of *Azospirillum* sp. B510, and may have been acquired from Beta-proteobacteria.

T6SS are involved in a broad variety of bacterial functions: from pathogenesis (by delivering effectors to target eukaryotic cells) to biofilm formation and stress sensing [64]. This large set of functions is reflected by a vast diversity of regulatory mechanisms [65]. T6SS can also confer toxicity towards other bacteria, providing a means of interspecies competition to enhance environmental survival [66]. Upregulation of *A. brasilense* Sp245 T6SS in response to exposure to IAA, as could happen in the rhizosphere, favors a role in plant-bacteria interactions [67]; the role of T6SS thus deserves to be investigated.

Several ORFs in all four *Azospirillum* genomes have been annotated as laccase-like; however, only one ORF (AZOLI_p30139 in *A. lipoferum* 4B and AZL_c02540 in *Azospirillum* sp. B510) possesses two typical copper-binding motifs [68]. Laccases- or laccase-like multicopper oxidases (EC 1.10.3.2) catalyze the oxidation of various substrates, such as phenols, diamines and metals, coupled with the reduction of molecular oxygen to water. The first report of a prokaryotic laccase is from *A. lipoferum* 4B [69], where it was shown to play a role in melanization and utilization of plant phenolic compounds [70]. Moreover, laccase-positive strains are less sensitive to the inhibitory action of quinone analogs due to rearrangements of their respiratory chain, a feature that might be a competitive advantage in the rhizosphere in the presence of quinone compounds [71]. A survey of bacterial laccases suggests they are an advantageous trait for a rhizosphere bacterium as they are involved in various functions such as copper resistance, manganese oxidation, pigmentation, oxidation of toxic compounds, and destruction of reactive oxygen species [72].

2.8. Accessory Components Related to Genome Plasticity

The extraordinary genome plasticity of *Azospirillum* has been evidenced by experimental data [14,73] and by whole genome alignments [12]. However, comparison of the AZO-core and the ANC-core does not allow the identification of key determinants that could partly explain this genomic plasticity, such as genes encoding recombinases, resolvases or topoisomerases.

Genomic regions carrying prophage elements seem to be specific to each strain; the only related element present in the AZO-core encodes a phage-related lysozyme (AZOLI_2690 / AZL_003440 / AZOBR_20012 / AZCBG_120001). This gene is absent from the ANC-core despite its initial assignment as ancestral [12]; this discrepancy comes from the fact that an orthologue is present in the genome of *Magnetospirillum magneticum* but absent from the genome of *R. centenum* used here to establish the ANC-core. The genomic context of this gene is identical in the four *Azospirillum* strains but does not display any other phage-related genes, suggesting that this gene is a phage remnant.

In *A. brasilense* Sp245, a unique region encompassing about 65 kb (from AZOBR_p340083 to AZOBR_p340194) contains several ORFs of phage origin and many ORFs encoding proteins of unknown function, and is framed by transposase/integrase. This region could correspond to the 65-kb prophage previously isolated from this strain [15]. This putative prophage of *A. brasilense* Sp245 shows no homology to a prophage sequence obtained from *A. brasilense* Cd, an observation which is consistent with the absence of hydridization signal previously reported [15]; in addition, this region has no equivalent in the genome of *A. brasilense* CBG497. Blast search in the genome of *A. brasilense* CBG497 using the sequence of *A. brasilense* Cd prophage reveals a single hit with the above mentioned ORF encoding a phage-related lysozyme (AZCBG_120001); no other ORF of phage origin

was found in the genome of *A. brasilense* CBG497. The release of phage particles upon induction by mitomycin C was previously reported for several strains of *Azospirillum* [15]. When the same procedure was applied to *A. brasilense* CBG497, no lysis was observed indicating that CBG497 hosts no mitomycin C-inducible prophage (data not shown). Whereas all phages from *A. brasilense* strains displayed genomes sizes from 62 to 65 kb, phages from *A. lipoferum* strains (including 4B) and from *Azospirillum* sp. B510 displayed a size of about 10 kb. Several pieces of evidence imply that these small prophages are rather gene transfer agents (GTA) than real prophages [15]. GTAs typically package bacterial genome fragments and atypically package a portion of their own genome and constitute conspicuous mechanisms of generalized transduction; they seem to be widespread among Alpha-proteobacteria [74]. However identification of GTA genes from sequenced genomes is not always straighforward as GTA genes can be scattered throughout the genome [74,75].

Two chromosomal regions with their putative *att* sites were previously identified as prophages in the genome of *Azospirillum* sp. B510 [10]: B510PP01, a region of 66.7 kb that is partially duplicated (60.2 kb) and B510PP02, a region of approximately 20 kb. Only the two ORFs framing B510PP01 (*i.e.*, AZL_008150 and AZL_008670) have orthologues in the *A. lipoferum* 4B genome (respectively, AZOLI_2072 and AZOLI_2071), suggesting that this phage may no longer be present in the latter strain. Blast searches with GTA genes of *Rhodobacter capsulatus* (accession number AF181080) identify putative GTA genes in the two duplicated sequences of B510PP01 (identity >25%); moreover, a stretch of four contiguous genes (encoding terminase / portal protein / prohead protease / capsid) display a similar organization than the corresponding GTA genes of *R. capsulatus*. Thus it is likely that B510PP01 or part of B510PP01 corresponds to a GTA.

Four prophage regions are present in *A. lipoferum* 4B. First, a chromosomal region of 31.4 kb (AZOLI_1757 to AZOLI_1794) harbours four genes (AZOLI_1775 to AZOLI_1771) that despite no homology at the DNA level display a similar organization with GTA genes of *R. capsulatus* encoding terminase, phage portal protein, phage prohead protease and capsid. This region could correspond to the phage particles containing random 10-kb fragments of host genomic DNA [15]. Three other regions bear chromids (AZOLI_p10448 to AZOLI_p10472, 25.5 kb; AZOLI_p10780 to AZOLI_p10794, 13.6 kb; AZOLI_p20026 to AZOLI_p20039, 13.1 kb) and have similarities with lambda-type or Mu-type prophages.The tRNAs lie upstream or downstream of these three regions.

So *A. lipoferum* 4B and *Azospirillum* sp. B510 have been subjected to multiple phage infection events, that may have contributed to genomic rearrangements. Moreover, GTA mediating generalized transduction may have contributed to acquisition of foreign DNA.

Other elements that certainly contribute to the high genomic plasticity of *Azospirillum* genomes are CRISPR sequences (*i.e.*, Clustered Regularly Interspaced Short Palindromic Repeats); CRISPR are thought to be involved in repartition of genome copies during cell division, to facilitate recombination and act as a defence mechanism against phages [76]. CRISPR were previously identified in 4B (126), B510 (153) and Sp245 (12) [12] and searched in CBG497 using the CRISPR web interface [77]. Seventeen CRISPR could thus be identified in the genome of *A. brasilense* CBG497 (on chromosome and on p2). Such smaller numbers of CRISPR in the genomes of *A. brasilense* strains might be due to their unclosed status or to their limited exposure to phage infections. Finally, a detailed analysis of the two closed genomes (those of *A. lipoferum* 4B and *Azospirillum* sp. B510) revealed the presence of multiple insertion sequences scattered in all the replicons [12]: 99 IS belonging to 37 different families

in *A. lipoferum* 4B and 310 IS belonging to 59 different families in *Azospirillum* sp. B510. Altogether, these features may have contributed to shape *Azospirillum* genome and to promote rearrangements between the different replicons.

3. Experimental Section

3.1. DNA Sequencing

DNA extraction of *A. brasilense* CBG497 and sequencing using the pyrosequencing method was performed as previously described [18]. 156 contigs were assembled into six replicons according to the genome organization of *A. brasilense* Sp245 using the MUMer software [78]. The sequence and annotations are available from the MicroScope platform [79,80].

3.2. Phylogenetic Analysis and Genome Comparisons

The 16S rRNA sequences were downloaded from EMBL. The sequences were first aligned using MUSCLE [81] and a maximum likelihood tree was generated using the SeaView platform [82]. To cluster protein families, BLAST-P comparisons of ''all *versus* all'' complete proteomes of *A. brasilense* CBG457, *A. brasilense* Sp245, *A. lipoferum* 4B and *Azospirillum* sp. B510 were done. Clustering was achieved with MCL using an e-value of 10^{-7} and an inflation parameter of 1.2 [22].

3.3. Orthologues Grouping and Analysis of Evolutionary Rates

The most probable set of orthologous proteins shared by the four *Azospirillum* strains (designated AZO core) was identified using a reciprocal best-hit criterion. To that end, all the predicted proteins of one genome were searched against the other predicted proteomes and vice versa using BLAST with cutoff e-value of 10^{-12} and employing the Blosum-80 matrix [83]. In addition to this criterion, to be included in an orthologue group, the alignment region between the subject protein and the query protein had to be at least 75%, and there had to be at least 35% similarity of both query and target sizes. 2,328 orthologue groups were identified in *Azospirillum*. Exclusive genes were recorded as those with no hit in the genomes at e-value of $<10^{-6}$. COG categories for each orthologue group were retrieved from the MaGe platform on the genome of *A. lipoferum* 4B; when several COGs were attributed, the COG with the highest score was retained. The same procedure was performed with an additional genome, that of *Rhodospirillum centenum* SW, to determine the ancestral core genome (designated ANC core). The number of nucleotide substitutions per synonymous site ''Ks'' and the number of nucleotide substitutions per non-synonymous site ''Ka'' were determined with the KaKs Calculator v1.2 software testing all different selection models and model averaging [84].

3.4. Bench Experiments

PCR amplifications were performed according to the *Taq* polymerase manufacturer (Invitrogen, Cergy-Pontoise, France) in 25 µL using 50 ng of template DNA. The amplification cycle consisted of an initial 5 min at 95 °C; 35 cycles of 30 s at 95 °C, 30 s at annealing temperature, and 30 s at 72 °C; followed by a final 7-min extension at 72 °C. Genomic DNA was extracted from bacterial cultures

grown for 20 h in LBm broth with a DNeasy Tissue Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The following PCR primers (synthesized by Invitrogen) have been used: F9504 (GATCAGGCCCAAGTCAACCT) and F9505 (GTTCAGCAGGTCCAGGATGT) for *vnfX* (AZOBR_p350013); F9510 (GCATGATCGTCTACACCACCT) and F9511 (GATGCCGTACTGCTTCTTCAG) for *vnfD* (AZOBR_p350020); F9536 (GTCAAGACCATGTCG AAGACC) and F9537 (GGAGTTCTTCAGCTCCAGGAT) for *vnfH* (AZOBR_p350022); F9528 (ATGGGGCTGGTGATCTTCTAC) and F9529 (GAAGATGCTGGTGAAGTCGAG) for AZOBR_p310195 (coding for a protocatechuate transporter).

For degradatation of aromatic compounds, precultures of *Azospirillum* were performed in AB malate 0.2% over 16 to 20 h at 28 °C under agitation. Cells were pelleted, washed and inoculated at an optical density of 0.05 into AB medium containing 0.1% phenylacetate or protocatechuate as sole carbon source. Stock solutions of aromatic compounds were prepared at 20% (w/v) in dimethyl sulphoxide. Growth was performed at 28 °C under agitation and monitored at 580 nm for the experiment with phenylacetate.

For utilization of IAA, cells were precultured and washed as above and were inoculated into AB medium containing 5 mM IAA as sole carbon source (stock solution of IAA at 0.5 M was prepared in acetonitrile). Growth was performed at 28 °C under agitation and recorded after three days.

Induction of phage particles by mitomycin C treatment was performed on *A. brasilense* CBG497 as previously described [15].

4. Conclusions

The genome of *A. brasilense* CBG497, a strain isolated from maize grown on an alkaline soil in the northeast of Mexico, was obtained and comparative analyses were performed with three *Azospirillum* genomes previously described. The four *Azospirillum* genomes studied here have genomes ranging from 6.5 to 7.6 Mbp, and are composed of six or seven replicons; chromids and plasmids comprise the largest proportion of the total genome (from 55.2% to 59.8%). The *Azospirillum* core genome consists of 2,328 proteins, representing between 30% to 38% of the total encoded proteins within a genome. It is mainly located on chromosome and contains 74% of genes of ancestral origin shared with some aquatic relatives. The non-ancestral part of the core genome is enriched in gene involved in signal transduction, in transport and metabolism of carbohydrates and amino-acids, and in surface properties, features linked to adaptation to soil and rhizosphere. However, many strain-specific or species-specific genes exhibit functions related to colonization of plant roots (chemotaxis, synthesis of surface polysaccharides, TAD pili), to plant-growth promotion (notably biosynthesis of hormones) and more generally to rhizosphere competence (catabolism of aromatic compounds, iron uptake). Thus, it appears that although *Azospirillum* strains harbour a common set of genes relevant for adaptation to the rhizosphere, each species or strain possesses unique genetic determinants, evidencing niche-specific adaptation. In addition, all genomes contain accessory components related to genome plasticity that could promote acquisition of foreign DNA or rearrangements between replicons. Transcriptomics approaches on *Azospirillum* during their interaction with host plants are now being developed in order to identify bacterial genetic determinants that are essential for this associative symbiosis.

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Annexe III

Tableau additionnel
Chapitre II, Partie 1

Fourni au format électronique

Supplementary Table S1. Significantly regulated genes (P_{adj}<0.05 and |FC|≥2) and bioprocess assignation.

Annexe IV

Tableau additionnel
Chapitre II, Partie 2

Fourni au format électronique

Tableau supplémentaire S4. Significantly regulated genes (P_{adj}<0.05 and |FC|≥2) and bioprocess assignation.

Annexe V

Tableaux additionnels
Chapitre III

Fourni au format électronique

Additional File 1.
Additional File 2.
Additional File 3.
Additional File 4.